VOL. XXXI[illegible] APRIL, 1917 No. 3

THE JOURNAL

OF

BIOLOGICAL CHEMISTRY

FOUNDED BY CHRISTIAN A. HERTER AND PUBLISHED BY MEANS OF THE CHRISTIAN A. HERTER MEMORIAL FUND

EDITED BY

H. D. DAKIN, New York, N. Y.
A. N. RICHARDS, Philadelphia, Pa.
E. K. DUNHAM, New York, N. Y.
DONALD D. VAN SLYKE, New York, N. Y.
LAFAYETTE B. MENDEL, New Haven, Conn.
CLARENCE J. WEST, New York, N. Y.

WITH THE COLLABORATION OF

J. J. ABEL, Baltimore, Md.
STANLEY R. BENEDICT, New York, N. Y.
R. H. CHITTENDEN, New Haven, Conn.
OTTO FOLIN, Boston, Mass.
WILLIAM J. GIES, New York, N. Y.
L. J. HENDERSON, Cambridge, Mass.
REID HUNT, Boston, Mass.
W. A. JACOBS, New York, N. Y.
WALTER JONES, Baltimore, Md.
J. B. LEATHES, Sheffield, England.
P. A. LEVENE, New York, N. Y.
JACQUES LOEB, New York, N. Y.
A. S. LOEVENHART, Madison, Wis.
GRAHAM LUSK, New York, N. Y.
E. V. MCCOLLUM, Madison, Wis.
A. B. MACALLUM, Toronto, Canada.
J. J. R. MACLEOD, Cleveland, Ohio.
JOHN A. MANDEL, New York, N. Y.
A. P. MATHEWS, Chicago, Ill.
P. G. HOYT, Ann Arbor, Mich.
THOMAS B. OSBORNE, New Haven, Conn.
T. BRAILSFORD ROBERTSON, Berkeley, Cal.
P. A. SHAFFER, St. Louis, Mo.
A. E. TAYLOR, Philadelphia, Pa.
F. P. UNDERHILL, New Haven, Conn.
V. C. VAUGHAN, Ann Arbor, Mich.
ALFRED J. WAKEMAN, New Haven, Conn.

PUBLISHED MONTHLY
BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH
FOR THE JOURNAL OF BIOLOGICAL CHEMISTRY, INC.
AT [illegible] MONUMENT AVENUE, BALTIMORE, MD.

Copyright 1917 by The Journal of Biological Chemistry

Entered as second-class matter, August 1, 1911, at the Post Office at Baltimore, Md., under the Act of March 3, 1879.

FREAS

Constant Temperature Water Bath

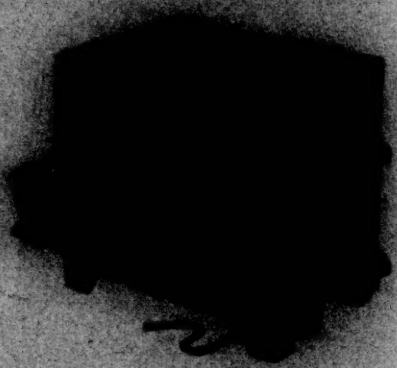
Consists of a copper tank with asbestos outer covering. The inner dimensions are 18x12x4 inches deep. The thermostat control is the Freas, already so successfully employed in the ovens and incubators. With this bath constant temperatures are maintained as desired from room temperature up to the boiling point of water.

Furnished either with or without cover

Price without cover - - - - - - $55.00
Price with cover, as shown by cut 65.00

Freas Sensitive Water Thermostat

Consists of copper tank with windows. Tank has asbestos outer covering and is provided with motor and stirring device. The thermostat control is the Freas bimetallic. The control is as accurate as with a mercury thermostat, while danger of breakage is removed and the construction is rendered much simpler. Adapted for a temperature range up to 60° C. Working space (above the wire screen) is 18x12x10 inches deep.



Price $115.00

EIMER & AMEND

ESTABLISHED 1851

Third Ave., 18th to 19th Sts.
NEW YORK CITY

Branch Offices and Salesrooms
2011 Jenkins Arcade, Pittsburgh, Pa.
45 Sparks Street, Ottawa, Canada

THE PARTITION OF NON-PROTEIN NITROGEN IN THE BLOOD OF FRESH WATER FISH.

By D. WRIGHT WILSON and EDWARD F. ADOLPH.

(*From the Laboratory of the United States Fisheries Biological Station, Fairport, Iowa.*)

(Received for publication, February 9, 1917.)

The introduction of microchemical methods has made possible many biological investigations which were formerly precluded by the difficulty of obtaining sufficient amounts of material. This has been the case with studies on the blood and urine of fish. The only accurate quantitative chemical data are found in the recent papers of Dr. Denis[1] on marine fish, where she reports results which indicate that the composition of the blood and urine is not only different from that of other animals but may even vary in different species of fish.

It has therefore seemed desirable to extend the studies to fresh water varieties in order to gain further insight into the processes of metabolism in these animals. The present study has included determinations of total non-protein nitrogen, urea, ammonia, amino nitrogen, creatine, and creatinine in the whole blood and in the plasma of several species of fresh water fish. The nitrogen partition and the distribution of materials between plasma and corpuscles displayed several unique relationships. The creatine content of the plasma was greater than that of the corpuscles. Urea was present in surprisingly small quantities in most of the bloods and was in greater concentration in the corpuscles than in the plasma. This unequal distribution is in distinct contrast with the uniform diffusion of the compound throughout mammalian tissues.

We have found that the amino nitrogen constitutes the major portion of the non-protein nitrogen in whole blood and is the largest single component in plasma. The quantities present are

[1] Denis, W., *J. Biol. Chem.*, 1912–13, xiii, 225; 1913–14, xvi, 389.

much greater than those found in mammalian blood. This is a complete reversal of the relationship between urea and amino nitrogen in the bloods of mammalia.

The following methods were employed. (1) Total non-protein nitrogen—Folin's method as modified by Bock and Benedict. (2) Urea—Marshall's method. On account of the low values obtained the method was checked in the usual ways including the addition of urea to the blood to make sure that the enzyme activity was not inhibited. The ammonia nitrogen has been subtracted. (3) Amino nitrogen—Van Slyke's method. The filtrate from the ethyl alcohol precipitation was used as well as portions of the filtrate from the total non-protein nitrogen determinations. There was no evidence of unusual amounts of amines or other nitrogenous compounds which react slowly with nitrous acid as the correction values were similar to those obtained with other bloods. (4) Creatinine—Folin's method. (5) Creatine—the procedure described by Denis[1] was used. The total non-protein nitrogen, urea, and amino nitrogen determinations were always carried out in duplicate, the others usually singly.

The blood was obtained from the living fish mainly from the caudal artery and vein by clipping off the tail and collecting the blood which dripped from the cut vessels. Some specimens were collected through a needle from the caudal vein while others were obtained by introducing a needle into the aorta. Potassium oxalate was used to prevent clotting. From 40 to 150 fish were necessary to furnish the desired amount of material for analysis.

To secure composite specimens of whole blood and plasma which might be entirely comparable, the blood was divided, soon after it was drawn, into two parts, one-third was put aside for the whole blood composite and two-thirds were centrifuged and the plasma was removed. The hematocrit readings were taken after centrifuging for 20 to 30 minutes. A Daland hematocrit was also used. The collections were kept in ice until sufficient quantities were obtained, when the various determinations were started. There was considerable variation in the ease with which unhemolyzed blood was obtained. No hemolysis was evident in the blood from the catfish and the croppie, while others showed slight hemolysis.

Little definite information was obtained as to the nutritive

condition of the fish. The carp and gar had been kept for some time in artificial ponds and fed regularly. The other fish were bled within a few hours after they had been brought in from the river. A considerable amount of food was found in the intestines of the catfish. The blood from the sturgeon appeared to contain a considerable amount of fat and that from the carp less.

The data obtained may be found in the accompanying table. The unusually small quantity of urea nitrogen in nearly all of the bloods is at once apparent. A large variation may, however, be

TABLE I.

Composition of Fish Blood and Plasma per 100 Cc.

	Non-protein N.		Urea N.		NH_3 N.		NH_2 N.		Creatinine.		Creatine as creatinine.		Hematocrit.
	Whole blood.	Plasma.	Whole blood.	Plasma.	Whole blood.	Plasma.	Whole blood.	Plasma.	Whole blood.	Plasma.	Whole blood.	Plasma.	
	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	per cent
Sturgeon (*Scaphirhynchus platorhynchus*).	29	22	1.1	0.4	1.7	1.0	17	10	2.5	1.0	3.5	2.8	30
	37	30	2.5*	1.8*	1.6				2.6		2.0		
Gar (*Lepisosteus platostomus*).	31	21	5.0	4.5	0.9	0.7	18	7	1.3	1.2			34
		22		5.2		1.3		13		1.5			
Carp (*Cyprinus carpio*).	42	40	2.3	2.0	1.3	0.5	34	18	1.3	1.3	4.1		39
			2.8*				18				5.0†	6.5†	
Croppie (*Pomoxis annularis* and *sparoides*).	46	35	3.0	2.5	0.9	0.9		13	1.2	1.4	9.2	12.7	30
							25	11					
Catfish (*Ictalurus punctatus*).	47	32	2.7	1.0	0.9	0.8	31	13	0.8	0.8	3.6	5.4	34
		41		1.2		1.3		14		0.8		4.0	
Sheepshead (*Aplodinotus grunniens*).	59	49	16.2	13.5	1.8	1.5	21‡	16‡	1.8	1.0	6.2	8.0	32
		41		9.5		1.2		11		0.9		6.9	

* Urea + ammonia N.

† Total creatinine.

‡ The figures in the horizontal columns are from determinations of a single composite specimen of blood and its plasma, except where noted by ‡.

noted. Whether this indicates fundamental differences in the fish or merely accidental variations due to the condition of nutrition or other factors cannot be decided from the data at hand.

The amounts of urea found in most of our experiments are similar to those observed by Karr and Lewis[2] in the blood of hens. The comparison is mainly of interest by way of contrast for it is well known that uric acid is the chief end-product of protein metabolism in the hen, while fish excrete only very small amounts. The urea in the urine of teleosts constitutes, however, a relatively low percentage of the total nitrogen.

We have found in only a single instance a concentration of urea as great as those reported for marine teleosts by Denis. It is interesting to note that the ganoids (sturgeon and gar) while perhaps more closely related to the marine elasmobranchs than are the teleosts, as indicated by their evolutionary development and cartilaginous skeletons, show no relationship so far as the urea concentration of the blood is concerned.

The variations in the content of urea in the blood from different families of fish exhibit an interesting parallelism with the differences in the osmotic pressures. The blood of the elasmobranchs, which contains relatively enormous quantities of urea (2 gm. per 100 cc.), has an osmotic pressure closely approximating that of sea water[3] ($\Delta = 2.3°$). In the marine teleosts, however, the blood contains but one-hundredth the amount of urea and has a much lower osmotic pressure (average $\Delta = 0.7°$). Our results would indicate that there is a tendency toward a lower content of urea in the blood of several fresh water fish, while the osmotic pressure is, in general, still less ($\Delta = 0.5°$). In connection with the differences in osmotic pressure in these animals it may be observed that the elasmobranchs are dependent upon the surrounding medium for the maintenance of the osmotic pressure of the blood, while the ganoids and teleosts seem to be partially independent of the medium. These facts raise several interesting speculations concerning the mechanisms involved in maintaining the osmotic pressure of the body fluids and the possibility of the diffusion of urea and other substances through the

[2] Karr, W. G., and Lewis, H. B., *J. Am. Chem. Soc.*, 1916, xxxviii, 1615.
[3] Bottazzi, F., *Ergebn. Physiol.*, 1908, vii, 161.

gills. The small amount of nitrogenous materials eliminated by the kidney has already been observed.

The extremely low content of urea in the blood of fresh water fish was further emphasized by the observation that the plasmas contained even less than the whole bloods. The localization of urea in the corpuscles is evident and quite unexpected. Analyses of tissues from various animals by Marshall and Davis,[4] Bang,[5] and Karr and Lewis[2] have shown that urea is uniformly distributed throughout the body, the same in plasma as in corpuscles, and have led to the assumption that urea may readily diffuse throughout the body (wherever sufficient water is present) and be maintained at a strikingly uniform concentration in all tissues. Our results argue against a too general acceptance of such an assumption. Furthermore, the data of Karr and Lewis show that the tissues of the hen may vary considerably in their content of urea. As mentioned above, the amounts found by these investigators in the blood and other tissues of the hen are similar to the quantities found by us in the blood of fish. Hence, it seems possible that there may be a tendency for the cells of the blood as well as other cells of the body to retain urea when its concentration becomes very low.[6]

The percentage of urea nitrogen to total nitrogen ranges from 4 to 27 in whole blood and from 2 to 29 in plasma. The majority of the values are surprisingly low when compared with other animals and at once lead us to inquire as to what types of nitrogenous compounds constitute the large remaining portion of the non-protein nitrogen.

Amino nitrogen was present in amounts which at once account for most of the non-protein nitrogen of the blood. There were found concentrations of 17 to 34 mg. of amino nitrogen per 100 cc. of whole blood, which constituted from 60 to 80 per cent of the total non-protein nitrogen.[7] These values are considerably higher

[4] Marshall, E. K., Jr., and Davis, D. M., *J. Biol. Chem.*, 1914, xviii, 53.

[5] Bang, I., *Biochem. Z.*, 1915–16, lxxii, 104.

[6] Data have been reported to indicate that under pathological conditions an unequal distribution of urea may be encountered.

[7] A few determinations carried out by the trichloroacetic acid precipitation as described by Bock indicated that the values reported above may be too low. A sample of the whole blood composite from the catfish gave results for both amino nitrogen and total non-protein nitrogen about 50 per cent higher than those tabulated above.

than the figures reported for amino nitrogen in the blood of mammals.[8] Van Slyke and Meyer[9] found 3 to 10 mg. in the blood of dogs, depending on the state of nutrition of the animals. Bock[10] has recently reported intermediate values for bloods of various mammalia.

The plasma contained less amino nitrogen than the whole blood. Calculated from the hematocrit values, the corpuscles were found to contain from three to five times as much as the plasma. György and Zunz[11] and Costantino[12] observed a similar massing of the amino nitrogen in the corpuscles of dogs, although the concentration was much lower in these animals.

The relationship between the amino nitrogen and urea is practically the reverse of that in the blood of higher animals where we find urea the chief nitrogenous constituent. The unusual concentration of amino nitrogen suggests that the amino-acids as such may play a more important part in the intermediary protein metabolism of the fish and possibly constitute an important means for the elimination of nitrogen. The small proportion of total nitrogen accounted for by the materials determined by Denis in the urine of fish (urea, ammonia, uric acid, creatinine, and creatine) lends support to the suggestion.

The relatively high content of ammonia in the bloods examined agrees with the observations of Denis on marine fish. We have not obtained, however, figures approaching the maximum values which she reported. The rapidity with which ammonia develops in blood on standing, together with the fact that several hours are unavoidably required to collect sufficient material for the experiments, would indicate that the minimal values probably represent more exactly the actual amounts of ammonia in the circulating blood.

The content of creatinine in whole blood and plasma was similar to that in mammalian blood. Creatine was present in somewhat larger quantities than has been found in the blood of higher animals by a similar method.[13] The preponderance of creatine

[8] Our figures range even higher than those reported by J. C. Bock, (*J. Biol. Chem.*, 1917, xxix, 191) for the amino nitrogen in the blood of birds.

[9] Van Slyke, D. D., and Meyer, G. M., *J. Biol. Chem.*, 1912, xii, 399.

[10] Bock, *J. Biol. Chem.*, 1916–17, xxviii, 357.

[11] György, P., and Zunz, E., *J. Biol. Chem.*, 1915, xxi, 511.

[12] Costantino, A., *Biochem. Z.*, 1913, lv, 402.

[13] Wilson, D. W., and Plass, E. D., *J. Biol. Chem.*, 1917, xxix, 413.

over creatinine in the plasma of fish corresponds to the relationship of the two compounds in the urine and emphasizes the relative importance of the former in the creatine-creatinine metabolism of these animals at least in so far as concerns excretion.

The variations in concentrations of materials between plasma and corpuscles have been demonstrated in many ways, especially in connection with inorganic constituents and lipoid materials. The importance of such study in connection with the nitrogenous substances has not been sufficiently emphasized. The known compounds making up the non-protein nitrogenous fraction of the blood are for the most part materials of interest chiefly as end-products ready for elimination from the body. The study of the distribution of these substances between corpuscles and plasma as well as between plasma and tissues may assist in solving some of the intricacies of the intermediary metabolism with which the various compounds are concerned.

SUMMARY.

The following constituents were determined in the whole blood and in the plasma of several species of fresh water fish including ganoids and teleosts: total non-protein nitrogen, urea, ammonia, amino nitrogen, creatinine, and creatine.

The urea content of most of the bloods was unusually low. The concentration in the plasma was less than that in the corpuscles.

The amino nitrogen constituted the major part of the total non-protein nitrogen of the blood. The corpuscles contained considerably more than the plasma.

Creatine was present in larger amounts in the plasma than in the corpuscles and was unusually high in the plasma.

CREATINE AND CREATININE IN WHOLE BLOOD AND PLASMA.

By D. WRIGHT WILSON and E. D. PLASS.

(From the Laboratory of Physiological Chemistry, Johns Hopkins University, and the Obstetrical Clinic, Johns Hopkins Hospital, Baltimore.)

(Received for publication, February 13, 1917.)

The methods elaborated by Folin[1] for the determination of small quantities of creatine and creatinine have been of great service in quantitative investigations involving these compounds, and the numerous criticisms and modifications suggested as improvements on the original technique are sufficient evidence of their widespread use. But recent discussions[2, 3, 4] have disclosed several possible sources of error which have been sufficiently emphasized to prevent further confusion.

During the last year we have been interested in the estimation of creatine and creatinine in blood and have encountered several of the difficulties described in recent communications. With these obstacles overcome to our satisfaction, we have determined preformed and total creatinine both in whole blood and in plasma from different animal species, using the original method of Folin and, at the same time, another procedure for total creatinine which we shall describe. Comparisons of the two methods have indicated that the method of Folin may yield inaccurate results for total creatinine, at least when applied to whole blood. The differences observed between the concentrations of creatine in whole blood and plasma from human beings as determined by the newer method emphasize the importance of such differentiation in studies whose object is to correlate the quantities of the

[1] Folin, O., *J. Biol. Chem.*, 1914, xvii, 475.

[2] McCrudden, F. H., and Sargent, C. S., *J. Biol. Chem.*, 1916, xxvi, 527.

[3] Hunter, A., and Campbell, W. R., *J. Biol. Chem.*, 1916–17, xxviii, 335.

[4] Folin, O., and Doisy, E. A., *J. Biol. Chem.*, 1916–17, xxviii, 349.

substance in blood and urine. A comparison between the concentrations of creatine and creatinine in the plasma and in the urine of the various animals studied has led us to the conclusion that a rough quantitative relationship exists.

The use of Folin's methods for the estimation of creatine and creatinine necessitates the comparison of slight colorations due to small amounts of creatinine in the presence of alkalinized picric acid solutions of relatively high concentration. As considerable errors occur whenever creatinine solutions of different concentration are compared, curves of correction similar to those recently described by Hunter and Campbell[3] were worked out. We have plotted the actual against the theoretical readings, at the same time plotting the logarithms of the concentrations of creatinine along the theoretical axis. The resultant curve fell away gradually from the straight line demanded by theory. Our readings are practically identical with those reported by Hunter and Campbell so that a repetition need not be made. In addition, we have worked out a curve for the 1 mg. standard in 1.2 per cent picric acid and find that our readings do not vary appreciably from the theoretical when reading stronger solutions against the standard, but when reading a solution containing 0.5 mg. of creatinine per 100 cc. against the 1 mg. standard set at 10 mm. an error of 1.5 mm. was observed. The shape of this curve is similar to the others. In working with diluted picric acid solutions, as described in Folin's micro methods for urine, we found no appreciable correction when comparing solutions up to 2 mg. per 100 cc. against the 1 mg. standard, and an error of only 0.5 mm. when comparing 0.5 mg. with the 1 mg. standard.

With the use of the curves described above, the elasticity of the methods is greatly increased in that the necessity for care in choosing standards is in large part obviated. We have demonstrated to our satisfaction many times that the applications of the considerable corrections necessary when reading dilute creatinine solutions in the blood determinations against standards of quite different concentration yield results agreeing with those obtained by comparisons with standards of proper concentration. With the application of these corrections and the personal correction, the colorimetric comparison takes on a greater accuracy than we are justified in assuming exists in the other parts of the methods.

The personal correction in colorimetric readings has recently been emphasized by Folin. It may be mentioned in this connection that our personal corrections were quite different. Under identical conditions, one of us has had a personal correction of 1 mm. and the other of 2 mm. when comparing a 0.2 mg. standard in both cells of the colorimeter. The intensity of the red color and, to some extent, the intensity of the light influence the magnitude of the correction.

Although saturated picric acid solutions are usually assumed to be 1.2 per cent, the solubility of the substance in water may vary from 1 to 1.5 per cent under ordinary changes of temperature in the laboratory. Such variations are sufficient to cause differences of 2 to 3 mm. in colorimetric readings when comparing 0.2 mg. standards. The presence of salts and perhaps of other impurities also influences the solubility. We have therefore thought it advantageous to use solutions of picric acid of known concentration and have made up roughly standardized solutions containing 1.2 per cent picric acid by titration with 0.1 N NaOH, using phenolphthalein as an indicator. Solutions of purified picric acid[5] of this concentration show some tendency to crystallize out at low temperatures while an ordinary preparation of sufficient purity does not. Crystallization may be retarded by introducing sodium chloride into the solutions.

We have observed that heat as well as light causes a decomposition of picric acid with the formation of compounds which yield a red color in alkaline solution. The deterioration of the picric acid solutions, when heated with other substances, is sufficient to affect materially the determination of small quantities of creatine. This being the case, the accuracy of the original method of Folin for the estimation of total creatinine in blood might well be questioned until confirmatory evidence is adduced by the use of another procedure. Hence, we have compared results obtained by the use of the original Folin method and by a procedure based on the removal of protein material by coagulation with heat and acetic acid. The method, described as follows, is merely a combination of several well known procedures: 10 cc. of blood are run into five volumes of boiling 0.01 N acetic acid in a casserole and the solution is boiled for about $\frac{1}{2}$ minute. An equal volume

[5] We have used a method for purifying picric acid similar to the one recently described by Folin and Doisy.[4]

of boiling water is added and the heating continued for about a minute. The solution is filtered through a folded filter and the casserole and paper are washed three times with 15 to 20 cc. of boiling water, rubbing free the material adhering to the dish. 10 cc. of a 15 per cent suspension of aluminium hydroxide are added to the filtrate which is then heated to boiling. Upon filtering again a water clear filtrate is obtained. This is acidified more strongly with 2 to 3 cc. of dilute acetic acid and evaporated on a water bath with a fan, keeping the dry sides of the evaporating dish well above the supporting rings. When concentrated to 2 to 5 cc., the solution is transferred to a 50 cc. Erlenmeyer flask, rinsing the dish several times with small portions of hot water and rubbing with a rubber tipped rod. The final volume of liquid is always approximately 10 cc. 1 cc. of 5 N HCl is added, the flask covered with a watch-glass, and heated on a boiling water bath for 3 to 4 hrs. After cooling, the solution is neutralized with 15 per cent NaOH and 20 cc. of 1.2 per cent picric acid are added. 1.5 cc. of 10 per cent NaOH are introduced, the solution is allowed to stand for 10 minutes, and then diluted to 100 cc. (or 50 cc.) with water. At the same time, 13 cc. of water and 20 cc. of picric acid solution containing a suitable amount of creatinine are introduced into a 100 cc. (or 50 cc.) volumetric flask, 1.5 cc. of 10 per cent NaOH added, and diluted to the mark with water after standing 10 minutes.

TABLE I.

Comparison of Acetic Acid Coagulation with and without Aluminium Hydroxide. Creatinine per 100 Cc.

Source.	Whole blood.		Plasma.	
	Acetic.	Acetic + Al $(OH)_3$	Acetic.	Acetic + Al $(OH)_3$
	mg.	*mg.*	*mg.*	*mg.*
Dog.....	2.0	2.0	2.5*	2.1
Cat.....	2.3	2.0	2.7*	1.9
Rabbit....	5.0 5.0 6.6*			
Pig 3.....	5.4* 6.7*	5.2 5.1	6.7*	5.8
" 4.....	4.8* 4.4	4.4 4.4	4.2	3.6
" 5.....	6.1* 6.5* 6.3* 5.9*	5.3 5.3 5.2		
Human...			1.8*	1.5 1.5 1.5

* Some pigment was observed.

When only limited quantities of blood were available, 5 cc. portions were used and the unknown and standard were diluted to 50 cc. It would seem, however, much more desirable to use quantities of blood which yield at least 0.2 mg. of creatinine. Curves similar to those mentioned earlier in this paper have also been worked out and used in these determinations.

The use of aluminium hydroxide is a distinct advantage though not absolutely necessary. The small amounts of protein which escape coagulation are removed by this means and the solution remains colorless even after heating with hydrochloric acid to convert creatine into creatinine. When a perfectly water clear filtrate results from the coagulation with acetic acid, little or no pigment may be formed during the conversion of creatine into creatinine, but usually a sufficient quantity is produced to interfere seriously with the determination. In Table I may be found the results of determinations made with and without the use of aluminium hydroxide on specimens of the same bloods. It will be noticed that whenever pigment formation was observed after the use of acetic acid alone, the results are higher than when aluminium hydroxide was also used. When no pigment was formed the results are the same. These observations, together with control determinations with creatine and creatinine solutions, show that the aluminium hydroxide does not remove appreciable quantities of either substance or interfere with the colorimetric estimation. The ease with which duplicates may be obtained with the use of aluminium hydroxide and the difficulties encountered when it is not used emphasize its value.

The method described above was used in connection with a comparative study on the creatine and creatinine content of whole blood and plasma from different animals. The preformed and total creatinine were also determined by the original procedure of Folin with and without preliminary hemolysis with water. The data which we have collected may be found in Table II. Most of the determinations on the blood from lower animals and a few of the human bloods were done in duplicate.

The results of the analyses of total creatinine in whole blood by the two different procedures show great divergence. In every case the picric acid method yielded values higher, at times

TABLE II.

Total and Preformed Creatinine in Whole Blood and Plasma by the Picric Acid and Acetic Acid Methods, in Mg. per 100 Cc.

Source.	Whole blood.					Plasma.				Remarks.
	Creatinine		Total creatinine.			Creatinine.	Total creatinine.			
	Not hemolyzed.	Hemolyzed.	Not hemolyzed.	Hemolyzed.	Acetic.	Picric.	Picric.	Acetic.	Creatine.	
Man (P.)	2.1	2.4	7.2	7.8		1.4	1.9			Normal.
" (P.)						1.3	2.0			"
" (M.)	2.1	2.9	9.2	11.0		1.4	2.2			"
" (W.)		2.3		5.5	2.9	1.4	1.8	1.4	0	Creatine-free diet. Normal.
Woman (H.)		3.1		6.5	3.0	1.2	1.8	1.2	0	Normal.
"		2.6		5.7	3.0	1.1	1.9	1.2	0.1	Mixed specimen. Normal.
" (T.)		1.2		6.8		1.1	1.6	1.6	0.5	In labor.
" (B.)						1.3	1.9	1.7	0.4	" "
" (S.)	1.8	2.5	4.9	5.5	3.8	1.1	1.6	1.3	0.2	Eclamptic.
Infant (M.)						1.1	1.9	2.0	0.9	New-born. (Serum.)
" (S.)						1.1	1.9	2.0	0.9	" "
" (B.)						1.0	1.9	2.0	1.0	" "
Dog 2	1.7	2.3	5.0	4.3	2.0	1.7	2.4	2.1	0.4	
" 3						1.0	1.5	1.3	0.3	
Cat 1	2.7	2.8	6.7	7.1		2.4	3.8	4.0*	1.4	Urine: creatinine 153 mg., creatine 21 mg. per 100 cc.
" 2	1.7	1.8	4.7	4.3	2.0	1.3	1.9	1.9	0.6	Fasted 24 hrs. Urine: creatinine 191 mg.; creatine 24 mg. per 100 cc.
Rabbit 1	2.4	3.8	7.5	7.7	5.0*	1.7	5.4		3.7	
" 2		3.1		10.5	8.1	2.0	8.5	8.3	6.3	Tartrate nephritis. Urine: 4 cc. contained 1.6 mg. of creatinine and 6.2 mg. of creatine.
Pig 2	2.0	2.1		6.5	4.7	1.9	5.3	5.2	3.3	
" 3		2.0			5.2			5.8	3.8	
" 4	1.8	2.0	6.0	5.8	4.4	1.8	4.0	3.6	1.8	
Hen 1	0.9	1.1	7.1	7.1	2.4*	0.8	2.3	2.6*	1.8	
" 2	1.2	1.2	5.0	5.1	2.4	1.2	2.4	2.5	1.3	

* No $Al(OH)_3$ used. Solutions colorless or nearly so.

100 per cent higher, than those of the acetic acid procedure. When applied to plasma the two methods were in closer agreement. Hence it would appear that something is present in the corpuscles which remains in the picric acid solution, so that after autoclaving an atypical color development is observed when the solution is made alkaline. We have often observed that the color development is much more rapid in these solutions than in the standard creatinine solutions and the development continues after the standard solution has nearly reached its maximum color (*i.e.*, after 10 minutes). Moreover, the color of the blood solution is different from that of the standard solution, possessing a definite brownish tinge and making the color comparison more difficult.

The application of the method of Folin to unhemolyzed plasma yields more satisfactory results. The color development is much more typical of pure creatinine solutions, and the agreement of the results with those of the acetic acid procedure, where the color development appears to be typical, lends support to the accuracy of the method. We believe that the quantities of total creatinine obtained by the use of the acetic acid procedure on blood should be considered maximum and not minimum values. It must therefore be concluded that the method of Folin is unsatisfactory for use with whole blood.

The rapid and continuous development of a color of different quality is also often noticed in the determinations of preformed creatinine, especially in whole blood. In one experiment, where actual comparisons were made, the creatinine concentration of whole blood, calculated from readings taken at the end of 10 minutes was 1.7 mg. per 100 cc.; 1 hour later, when read against the same standard, the calculated creatinine concentration was 2.5 mg., an increase of 50 per cent. The preformed creatinine values are usually higher when the blood is hemolyzed before the addition of picric acid. Whether this increase may be due to a liberation of creatinine which is otherwise occluded in the voluminous precipitate or merely the liberation of color-producing material not creatinine cannot be determined from the data at hand, but the latter assumption seems to us the more plausible.

Our observations on the preformed creatinine in the whole blood of different animals range from 1 to 3 mg. per 100 cc.,

quantities similar to most of those reported by other investigators. The blood of hens seemed to contain practically as much creatinine as that of other animals. This is contrary to the observations of Folin and Denis,[6] but more in accord with the data reported by Myers and Fine.[7]

The determinations of preformed creatinine in the whole blood are frequently higher than those in the plasma but the variations are sufficiently small to be ascribed to the anomalous color development when whole blood is used. It would seem probable that creatinine is equally distributed between plasma and corpuscles.

By the use of the acetic acid method described earlier in the paper, we have found from 2 to 5.2 mg. of total creatinine per 100 cc. in the whole blood of various animals which were presumably normal. Human blood contains about 3 mg. per 100 cc. These quantities are much lower than practically all of the reported data, for the reason that the picric acid method has been almost universally used. Shaffer[8] reports a single determination on the whole blood of a dog by a method of coagulation with heat and acetic acid. As he obtained 7.4 mg. per 100 cc., we should conclude that probably pigments interfered with the accuracy of the determination. Beker[9] analyzed the whole blood from three species of animals by coagulating with heat and acetic acid, precipitating with lead acetate, concentrating, and autoclaving with HCl. His results are much more in accord with ours. He found from 1.9 to 2.4 mg. per 100 cc. of dog blood, 2 mg. in pig blood, and from 1.9 to 2.7 mg. in ox blood.

In most instances, the plasma contained practically the same quantity of total creatinine as the whole blood. The human plasma, however, contains less. It appears that the corpuscles in human blood contain appreciably more total creatinine than does the plasma. Owing to the lack of entirely satisfactory data on preformed creatinine especially in whole blood it is impossible to say definitely whether the material stored in the corpuscles is creatine or creatinine. It would seem most prob-

[6] Folin, O., and Denis, W., *J. Biol. Chem.*, 1914, xvii, 487.
[7] Myers, V. C., and Fine, M. S., *J. Biol. Chem.*, 1915, xxi, 583.
[8] Shaffer, P. A., *J. Biol. Chem.*, 1914, xviii, 525.
[9] Beker, J. C., *Z. physiol. Chem.*, 1913, lxxxvii, 21.

able, however, that creatinine is present in practically equal amounts in corpuscles and plasma but the corpuscles may store some creatine under normal conditions.

The determinations of preformed creatinine in plasma seem to be sufficiently accurate to warrant a consideration of the quantities of creatine calculated by subtracting the values of the preformed from the total creatinine as determined by the acetic acid method. These figures are also tabulated.

A comparison of the relationship between creatine and creatinine in plasma and in urine seems to offer more convincing results than any such comparison of values obtained by the older methods on whole blood. We have found little or no creatine in adult human plasma. This observation offers an explanation for the fact that creatine is seldom found in the urine of normal men and in small and variable amounts in the urine of normal women. Whether the method is sufficiently accurate to show the presence of creatine in the plasma when, and only when, small quantities are eliminated is uncertain, but our few determinations point to that possibility. Infants, who are known to eliminate relatively large quantities of creatine, have in their plasma practically as much creatine as creatinine.

The quantities of creatine in the plasma of the dogs which we have examined are barely appreciable. The urine from these animals was not examined, but it is well known that creatine is excreted irregularly by normal dogs. More creatine was found in the plasma of cats, one of which had fasted 24 hours. A small amount of creatine was present in the urine of these animals.

A still larger quantity of creatine was found in the plasma of a rabbit. The animal was presumably normal but its nutritive condition was not determined. The recent work of Underhill[10] shows the sensitiveness of these animals to conditions causing the excretion of creatine. The second rabbit had received sodium tartrate several days previously but was not anuric. Daily examinations of the urine showed only a slight diminution in the output of creatinine, while the concentration in the blood was practically unchanged. There seemed to be, however, a definite accumulation of creatine resulting in a correspondingly greater

[10] Underhill, F. P., *J. Biol. Chem.*, 1916, xxvii, 127.

excess of creatine over creatinine in the plasma. A similar relationship was observed in the urine in which the creatine constituted over three-fourths of the total creatinine.

The different specimens of plasma from pigs contained different concentrations of creatine. We observed variations from 1.8 to 3.8 mg. per 100 cc. of plasma, quantities equal to or greater than the preformed creatinine. Here, again, the possible relationship of the quantities of creatine in the blood and urine is of considerable interest. McCollum and Steenbock[11] have shown that the pig normally excretes creatine and the amount eliminated is dependent to a considerable extent upon the protein ingestion. They have observed that creatine may constitute as much as 40 per cent of the total creatinine eliminated. The large and variable quantities of creatine eliminated by the pig may be dependent upon similar variations in its concentration in the plasma.

Creatine is present in larger quantities than creatinine in the plasma of hens, but the excess is not so great as might be expected from the analyses which have been reported on the urine of birds. Paton[12] states that "creatine takes the place of creatinine in the urine of the bird," but leads the reader to conclude that he may have detected small amounts of creatinine which were assumed to be formed by his analytical procedure. Thompson[13] has recently reported analyses of the urine from ducks, which would indicate that the creatinine elimination is not negligible but is from one-third to one-fifth as much as the creatine. While these observations support Paton's general conclusion, the elimination of creatinine as well as creatine in the urine is more in accord with our point of view that the presence of appreciable quantities of creatine or creatinine in the plasma leads to their excretion.

The results which we have obtained, first, by the examination of unhemolyzed plasma and, second, by the substitution of a more exact method for estimating total creatinine permit a much more exact and rational viewpoint of the quantitative relationships

[11] McCollum, E. V., and Steenbock, H., *J. Biol. Chem.*, 1912–13, xiii, 209.

[12] Paton, D. N., *J. Physiol.*, 1910, xxxix, 485.

[13] Thompson, W. H., *J. Physiol.*, 1916, l, p. xxii.

between creatine and creatinine in the blood and urine. The older observations led to the anomalous conclusion that creatine is present in considerable quantities in the blood of man while little if any normally escapes into the urine, but that under certain slightly abnormal conditions it may be eliminated in considerable quantities. Moreover, the variations in the concentration of creatine in the bloods of different animals showed no relationship to the relative amounts of creatine excreted. Our results would indicate that when appreciable quantities of creatine (or creatinine) are present in the plasma, some is eliminated in the urine and the amount eliminated bears a rough quantitative relationship to the concentration in the plasma. Such an hypothesis seems to be capable of satisfactory proof with the use of the procedures which we have employed in this investigation. Our observations also suggest that, as the concentrations of creatine in plasma and corpuscles of man are different, they should be studied independently.

SUMMARY.

The method of Folin for the determination of total creatinine is unsatisfactory for use with whole blood. A different procedure is suggested.

Data are presented for preformed and total creatinine in whole blood and plasma from different species of animals.

Adult human plasma contains only traces of creatine. Appreciable quantities are present in the plasma of infants. The plasmas of the pig and hen contain larger quantities.

A characteristic relationship appears to exist between the concentration of creatine in the plasma and its elimination in the urine.

STUDIES IN BENCE-JONES PROTEINURIA. II.

By A. E. TAYLOR, C. W. MILLER, and J. E. SWEET.

(*From the Department of Physiological Chemistry, University of Pennsylvania, Philadelphia.*)

(Received for publication, February 2, 1917.)

Elimination of the Protein.

A striking feature of Bence-Jones proteinuria is the free passage of large quantities of the protein through a kidney which holds back the normal serum proteins.

Most previous investigators have found the kidney of animals impervious to the Bence-Jones protein, although Boggs and Guthrie[1] found that sometimes rabbits excreted it after intravenous injection. We injected subcutaneously 40 cc. of Bence-Jones urine into each of two dogs, weighing respectively 8 and 9.5 kilos. All urine passed during the following 3 days was entirely free from protein of any kind (ferrocyanide and acetic acid, heat, HCl). On the 3rd day one of the dogs was etherized and into the femoral vein were injected 30 cc. of Bence-Jones urine. The urine of this dog for the next 2 days showed no protein of any kind. The amounts of Bence-Jones urine taken for injection represented in each instance a little more than 1 gm. of the protein.

To try the effect of larger quantities we etherized a 14 kilo dog and injected into a vein of the leg 5 gm. of Bence-Jones protein dissolved in 200 cc. of slightly alkaline Ringer's solution. The urine of this dog before injection showed just a trace of albumin; but as this is not uncommon in dogs and as the animal was strong, active, and apparently perfectly well, we did not reject him on that account. When coming out of the ether the animal urinated, and again about 20 minutes later. Both of these urines were entirely similar to that obtained before injec-

[1] Boggs, T. R., and Guthrie, C. G., *Am. J. Med. Sc.*, 1912, cxliv, 803.

tion, and free from Bence-Jones protein. The urine found the next (2nd) day produces a heavy white cloud when heated with a drop of acetic acid; this cloud almost disappears upon boiling and reappears upon cooling, and this can be repeated. The characteristic acid reactions are also given in strong typical fashion.[2] Evidently we here have a marked excretion of Bence-Jones protein. Upon estimation it was found that the urine contained 0.16 per cent, or for the total amount of urine 1.47 gm. of Bence-Jones protein. When the urine was carefully coagulated at boiling, allowed to cool, and filtered, the clear filtrate (which could no longer at any reaction or temperature be made to give cloudiness on heating) gave some opalescence when saturated with ammonium sulfate; and also gave moderate reactions with ferrocyanide and Roberts' reagent. While these positive tests in the filtrate may have been caused by failure to remove all of the coagulable protein, it seems more probable that there was a small amount of proteose present. Whatever the nature of this filtrate protein, its total amount was evidently very small in comparison with the coagulable protein. The urine found on the 3rd day was nearly free from protein. Spiegler and ferrocyanide tests both indicated traces, but these reactions were little if any stronger than this dog normally showed; there was no reaction for Bence-Jones protein. The urine found on the 4th day showed only traces of albumin, no stronger than before the experiment, so the dog was removed from observation. It is evident that rather less than one-third of the injected Bence-Jones protein was eliminated during the first 24 hours, and that after that time practically no more was recovered. Even granting that a very small amount of proteose was present in the urine of the first period, not more at most than one-third of the protein is accounted for; what became of the other two-thirds we are unable to say; but as even when injected directly into the blood stream it was neither eliminated as Bence-Jones protein nor digested and eliminated as proteose, it does not seem unreasonable to assume that it is promptly fixed by the blood cells or combined with something in the plasma, and subsequently made use of or catabolized in the organism.

When the dog has previously been moderately poisoned by uranium nitrate, the injection of considerable quantities of the

[2] Taylor, A. E., and Miller, C. W., *J. Biol. Chem.*, 1916, xxv, 281.

protein produces very different results. A healthy dog weighing 6.2 kilos was injected subcutaneously with 0.03 gm. of uranium nitrate, 4.8 mg. per kilo. By the next (2nd) day the urine showed a moderate amount of albumin, and on the 3rd day the dog received (under ether anesthesia) into a vein of the leg 8 gm. of Bence-Jones protein dissolved in quite alkaline Ringer's solution. After coming out of the ether he vomited twice. The filtrate of this vomitus could not be made to coagulate by heat and seemed free from Bence-Jones protein. The urine found on the 4th day contained some albumin by boiling, but hardly as much as before the protein was injected. There was no trace of coagulation when heated for an hour at 58° with a drop of weak acetic acid, and the HCl test was negative; evidently no Bence-Jones protein was present. There was, however, a very heavy reaction to ferrocyanide and acetic acid totally out of proportion to the amount of protein coagulated on boiling. The urine found on the 5th day had the same characteristics, and as the dog seemed to be moribund he was killed with chloroform, and about 30 cc. of urine were taken from the bladder. This gave the same reaction as before. Macroscopic examination of the stomach, liver, and gall-bladder revealed nothing, while the kidneys showed moderate inflammation.

This experiment was repeated substituting Bence-Jones urine for the separated protein. A dog weighing 12 kilos was injected subcutaneously with 0.075 gm. of uranium nitrate, 6.2 mg. per kilo. The next (2nd) day the urine contained a moderate amount of albumin by the heat, Spiegler, Roberts', and ferrocyanide tests. The urine found on the 3rd day reacted similarly and under ether anesthesia the dog received into a vein of the leg 250 cc. of Bence-Jones urine, representing nearly 7 gm. of protein. This dog also vomited after the ether, and the vomitus was, like that of the previous animal, free from Bence-Jones protein. The urine of the 4th day contained less albumin than before and no Bence-Jones protein. The reaction to ferrocyanide is very strong, and there is also a strong biuret test. To one part of the urine were added ten parts of absolute alcohol; this was allowed to stand an hour and filtered. To a portion of the filtrate was added water, evaporated down to small volume on the water bath, when it gave a strong biuret reaction. The

urine of the 5th day gave the same result. Also a portion of this urine was saturated with ammonium sulfate, and a drop of acetic acid added; the result was a heavy precipitate, and the clear filtrate gave a strong biuret reaction. Another portion of the urine was saturated with ammonium sulfate and allowed to stand with excess of the sulfate for 36 hours, when as before the clear filtrate gave a strong biuret reaction. The precipitate produced by the ammonium sulfate was in each instance many times heavier than the heat coagulum. The 6th day the dog died.

From these two experiments it would seem that the uranium dogs have lost their normal power of utilizing the Bence-Jones protein, and that the latter is so energetically hydrolyzed that a portion even escapes precipitation by ammonium sulfate.

It might be objected that the uranium itself or the etherization were in some way responsible for the appearance of digestion products in the urine. In order to test this point we injected another dog with uranium nitrate as before, but gave no protein. The urine for 2 days following showed no proteose. On the 3rd day the dog was etherized for 20 minutes, and the urine for the following 3 days still showed no proteose. This dog was the 14 kilo animal previously used in the experiment of injecting 5 gm. of protein. He was, however, entirely well as far as could be observed, and in the best of spirits. The amount of uranium nitrate used was 0.09 gm., 6.4 mg. per kilo; and it is interesting to note that although more uranium was used than in the other experiments this dog appeared to have practically recovered by the 6th day, the albumin having almost disappeared from the urine, and he was in the best of spirits with excellent appetite. He remained under our observation for a couple of weeks and was apparently absolutely well.

Another dog weighing 9.7 kilos was injected with uranium nitrate, using 0.06 gm., 6.1 mg. per kilo. On the 2nd day there was considerable albumin, and on the 3rd day also. On this day under ether anesthesia we injected into a vein 200 cc. of normal urine. The urine of the 4th, 5th, and 6th days showed no proteose. This animal recovered rather slowly, and it was 10 days before he again seemed in normal condition.

DISCUSSION.

These experiments indicate that the normal dog is able to utilize or catabolize a moderate amount of Bence-Jones protein, even when this is injected rapidly into the circulation; but that a limit is soon reached beyond which the excess protein is promptly excreted in unchanged condition. In dogs suffering from moderate uranium poisoning this power of utilization appears to be lost; though on the other hand no Bence-Jones protein is excreted as such, but only after being broken down to proteose. It is interesting that the dog receiving the largest dose of uranium (6.4 mg. per kilo) but no urine or Bence-Jones protein promptly recovered; that the dog receiving uranium nitrate and then normal urine showed more serious toxic symptoms but did recover; and finally, that of the two dogs receiving uranium nitrate and then Bence-Jones protein and freely excreting proteose, one died on the 6th day, and the other was moribund when he was killed on the 5th day. Since the Bence-Jones protein is not toxic to the healthy dog,[2] and since, apart from our own results, this amount of uranium would not be expected to cause rapid deaths,[3] it would seem that the uranium causes some change in the metabolic power such that the normally harmless or perhaps useful protein rapidly becomes hydrolyzed with unfavorable results. When dealing with uranium poisoning, one's attention is naturally directed toward the kidney; but in these experiments the peculiar effect of the uranium on the elimination of the protein might depend rather on a general toxic influence; possibly some powerful ferment may have appeared in association with the uranium intoxication, with the result of protecting the organism from the breakdown products of the poisoning; and this ferment, while successful in saving the animal from the uranium, is so active that as soon as an abnormal protein is introduced it causes him to be overwhelmed by proteoses formed from the injected protein faster than they can be eliminated. This is the

[3] Thus W. de B. MacNider (*J. Pharm. and Exp. Ther.*, 1913, iv, 491) in his studies concerning elimination by the uranium kidney after anesthetics and diuretics found that it required 6.7 mg. per kilo injected twice to cause the "desired changes in the kidney without the undesirable gastro-intestinal complications."

general type of explanation one would formulate on the basis of Vaughan's hypothesis.

It will be observed that the healthy dog which eliminated a portion of the injected Bence-Jones protein received only 0.36 gm. of the protein per kilo, whereas the uranium dogs received respectively 1.29 and 0.58 gm. per kilo; so there is here at least no evidence in favor of the supposition that a kidney permeable to normal serum proteins is on that account more easily passed by the Bence-Jones protein. On the other hand, the conditions of uranium poisoning are not sufficiently similar to those of a chronic nephritis to make positive statements, and we have not succeeded in finding a dog with a natural nephritis upon which this question could be more exactly studied.

The ease with which Bence-Jones protein passes through a kidney impermeable to the normal proteins is almost certainly not due to the molecule being small in size, since nearly all the more recent studies indicate that we have to deal with a higher protein of large molecular weight. For example, Hopkins and Savory, after careful amino-acid determinations state as their opinion[4] that: "Bence-Jones protein yields all those amino-acids which are to be obtained from typical proteins, and is therefore not a fractional product arising from the partial breakdown of protein in metabolism;" and this view is entirely in accord with the conclusions previously arrived at[2] on other grounds by two of us. What may be the nature of the physicochemical adjustment permitting this selective action on the part of the kidney we venture no surmise.

Origin of the Protein.

Two sources suggest themselves: The protein may be a special product of the tumor cells of the myeloma; or it may be a normal or possibly aberrant stage in the synthesis of some body protein, the completion of which is interfered with by deficiency of some necessary condition.

The first of these assumptions is doubtless the more natural, although there seems little direct evidence to support it. If the protein is in fact the product of the tumor cells we should expect to

[4] Hopkins, F. G., and Savory, H., *J. Physiol.*, 1911, xlii, 189.

find at least small amounts of it in the diseased marrow. Reach[5] examined the bones with negative results, but found the protein in the spleen, which organ contained a neoplasm similar to those found in the bones. However, Hopkins and Savory[4] found no trace of the protein in the bone marrow; and when the marrow was injected into rats none of the protein was found in the urine of these animals. When our case came to autopsy we were permitted to take only small specimens of the bones for microscopic examination, and were therefore not able to test this question ourselves. Rosenbloom[6] has suggested an osseo-albuminoid origin for the protein. The strongest argument in favor of the protein being a special product is the unusual amino-acid composition, the phenylalanine and tyrosine together amounting to over 9 per cent of the whole, which, as Hopkins and Savory point out, is "considerably higher than any yet described for a blood or tissue protein. Judging, indeed, by our figures as a whole, the protein would seem to stand by itself." On the other hand, the surprisingly large amounts excreted would rather indicate some more general source. As was previously pointed out[2] the biologic reactions of this protein relate it closely to the normal serum proteins, a conclusion further substantiated by Massini's complement fixation experiments.[7] Our ignorance of tumor composition and of their products, if any, or of the exact degree of foreignness experimentally limiting biologic reactions, makes it impossible to be dogmatic; but the conclusion may well be reserved whether a protein behaving biologically as does this is really a special, and to the organism previously unknown, substance.

If we were to assume that in the diseased marrow something was lacking which prevented the complete synthesis of a normal body protein, then by supplying to Bence-Jones protein normal bone marrow and excess of amino-acids we might, theoretically at least, observe the building up of a protein of higher coagulation point. We at first proposed to carry out such an experiment by perfusing normal dog bone with a solution of Bence-Jones protein and amino-acid solution. After a large amount of

[5] Reach, F., *Deutsch. Arch. klin. Med.*, 1905, lxxxii, 390.
[6] Rosenbloom, J., *Arch. Int. Med.*, 1912, ix, 236.
[7] Massini, R., *Deutsch. Arch. klin. Med.*, 1911, civ, 29.

preliminary experimentation we were forced to abandon this, as we were unable to devise and carry out any scheme that gave promise of reliable quantitative results; and since in this experiment we are sure to have both Bence-Jones and normal protein, it is necessary to operate quantitatively in order to learn whether the latter protein is being increased at the expense of the former. We then tried the following experiment *in vitro*. An amino-acid solution obtained by tryptic digestion of dog muscle was heated to boiling, filtered, and cooled, and to 200 cc. of it were added 2.25 gm. of Bence-Jones protein as well as the ground up bone marrow from the long bones of a large dog, removed as aseptically as possible and used perfectly fresh. A portion of this mixture was put into the incubator with toluene, and the other portion used to estimate under definite previously determined salt and acid relations the respective content in Bence-Jones and high coagulation point protein. The portion in the incubator was allowed to remain 7 days, then removed and analyzed as before. The results showed that only a trace of the Bence-Jones protein remained, but that nearly all of the serum protein had also disappeared. Therefore any possible synthetic action was entirely lost in the active digestion which had broken down nearly all of the coagulable protein. When we remember the comparative ease with which various ferments accomplish digestion and the well known difficulties surrounding the production artificially of biologic synthesis, this result will cause no surprise; and we feel that this experiment can only be regarded as leaving the question unsettled.

Course of the Case Previously Reported.

The improvement in the condition of the patient, previously reported,[2] up to March 1, 1916, proved to be only temporary. After this he failed at first slowly, then more rapidly, all the while maintaining the excretion of Bence-Jones protein at and finally above the previous high level. In addition there were soon added at first small then larger quantities of serum albumin. As we were unable to devise a process for separating with any precision the two kinds of protein without at the same time causing material denaturation, this change in the character of the excretion unfortunately compelled us to abandon a series of ex-

periments which had been planned along biologic lines to obtain more exact information as to which of the proteins of the normal organism the Bence-Jones body is most closely related to. The patient died in October, 1916, and for several months before that time no work was done by us except an occasional examination of the urine.

Autopsy.—The only noticeable abnormality of the viscera was several old healed lesions of the lungs. The pleural cavities contained considerable clear straw-colored fluid. The long bones were not affected; but in the bones of the pelvis, ribs, and vertebræ the marrow occupied a much greater space than usual with marked thinning of the walls; the body of the sacrum was so spongy as scarcely to resemble bone at all, and the bony structure of the ribs in some places was not over 0.5 mm. in thickness. The subsequent histologic examination showed undoubted myeloma, a fact of interest to clinicians since the x-ray had indicated no osseous abnormality.

Besides the specimens for histologic examination, which will elsewhere be reported, we obtained urine from the bladder, blood from the heart, and fluid from the pleural cavities. The *urine* presents the same depth of color and other appearance as usual, but contained 3.6 per cent of Bence-Jones protein, which was more than we had ever before found; and there was also considerable albumin. The *pleural fluid* gave well marked reactions for Bence-Jones protein, and upon estimation was found to contain 0.11 per cent. The *blood* after rubbing down the clot with water and pressing through the filter gave a well marked test with HNO_3 and heat,[2] also the filtrate from boiling coagulation clouded strongly upon cooling. The amount of blood available was not sufficient to make satisfactory determinations, but the protein appeared to amount to about 0.2 per cent, which is confirmed by the fact that the qualitative tests were definitely stronger than in the case of the pleuritic fluid similarly diluted. Because hemoglobin is coagulated or decomposed at about the same temperature at which Bence-Jones protein coagulates, the latter cannot be estimated in the presence of the former by the usual method; it is necessary to bring the whole to boiling and then filter through a hot filter and weigh the protein which separates on cooling. This process requires a large amount of material, gives

results which do not agree well, and apparently the figures are always too low.

From these autopsy findings it is clear that the protein circulates freely throughout the body.

SUMMARY OF BOTH PAPERS.

1. Brief clinical notes of the case are given.

2. A number of reactions of the Bence-Jones protein found in this case, together with methods of identification, are described.

3. A method of separating the protein and of preparing a protein-free urine is also described.

4. Anaphylactic sensitization is accomplished best by the separated protein, less well by the same amount of protein as found in the native urine. Some factor exists in the native urine which prevents the protein contained in it from exerting its normal sensitizing action; and this factor is thermostabile at 55°, but is destroyed by heating to near the boiling point or by such chemical manipulation as described in our method for preparing protein-free urine. Subsequent anaphylactic intoxication is shown equally by the native urine and by the separated protein.

5. The protein shows no direct toxicity.

6. The separated protein is digested with great ease by both pepsin and trypsin.

7. The protein is no proteose, but a higher protein of definite biologic stamp.

8. It is of endogenous origin, and might be derived either from the tumor cells of the myeloma, or produced through an interrupted or aberrant synthesis of some normal body protein. The biologic indications of close relationship to the normal blood proteins, and the enormous quantities produced would seem to favor the second alternative. The question remains obscure.

9. When we allowed Bence-Jones protein, normal bone marrow, and an amino-acid solution to remain together for 7 days nearly all coagulable protein had disappeared. In the presence of this active digestion we were unable to learn whether any synthesis had occurred or not.

10. Normal dogs can utilize or catabolize moderate quantities of Bence-Jones protein, but a limit is soon reached beyond which the protein is promptly excreted in unchanged condition.

11. In dogs suffering from moderate uranium poisoning this power of utilization is lost, and the Bence-Jones protein is energetically hydrolyzed and eliminated as proteose.

12. Moderate doses of uranium nitrate which provoke only moderate symptoms rapidly become fatal when Bence-Jones protein is injected into the circulation. An explanation of this in harmony with the Vaughan hypothesis would be that some ferment arises in association with the uranium intoxication protecting the organism from breakdown products of the poisoning, and that the ferment is so active that as soon as an abnormal protein is introduced the animal is overwhelmed by the proteose formed from the injected protein.

13. At autopsy the bones of the pelvis, ribs, and vertebræ were found involved in extensive myeloma notwithstanding that the clinical findings during life were negative.

14. Urine taken from the bladder at autopsy showed 3.6 per cent of Bence-Jones protein, besides considerable albumin; the pleural fluid showed 0.11 per cent of Bence-Jones protein; and the blood appeared to contain about 0.2 per cent of Bence-Jones protein. Evidently the protein circulates with great freedom throughout the body.

THE DETERMINATION OF CHOLESTEROL IN BLOOD.

By W. R. BLOOR.

(*From the Laboratories of Biological Chemistry, Harvard Medical School, Boston.*)

(Received for publication, February 27, 1917.)

Weston[1] has recently reported results obtained by the use of my method for the determination of cholesterol in blood. These results are inconsistent and justify his finding that in his hands the method was useless. On the other hand, the method has given consistent results in this laboratory in various hands for over a year and is known to be in use in other laboratories where a thorough testing out before adoption would be a matter of course. In published work on the subject Mueller[2] has obtained results similar to mine in a comparison of the method with that of Autenrieth and Funk and has reported the alcohol-ether method of extraction "most excellent." Denis[3] has reported essentially the same values for normal blood as I had obtained. While it could hardly be expected that any method would find acceptance at all hands, this method seemed to have the advantages of simplicity in principle and ease and rapidity in operation, with the further advantage that the same alcohol-ether extract may be used for the determination of other blood lipoids. It was desirable therefore to find out if possible the reason for Weston's inconsistent results, both for the justification of those already using the method and to correct if necessary the directions so as to prevent further trouble with it.

Experience has taught that in cases of this kind the most frequent cause of failure is a use of the method in some other way than that directed or intended. It is a fact well known to chemists that few methods are flexible enough to be used in any

[1] Weston, P. G., *J. Biol. Chem.*, 1916–17, xxviii, 383.
[2] Mueller, J. H., *J. Biol. Chem.*, 1916, xxv, 549.
[3] Denis, W., *J. Biol. Chem.*, 1917, xxix, 93.

other way than that worked out by the originator. While Weston's description of his procedure is brief and lacking in essential details, there is sufficient to show that he has probably violated both the principle and the expressed directions. Thus in his version of the method, after describing the extraction with alcohol-ether, bringing to 100 cc., and filtering, the filtrate was divided into two parts, the first part was evaporated to dryness, and the residue "taken up with chloroform." The second was treated with potassium hydrate and then as in the Weston-Kent method. Two points may be noted here; first, that the filtrate is not the whole extract—it is an indefinite volume depending on the amount held back by the precipitate and filter paper, and generally about 90 cc.—so that what is obtained by taking half of it is about 45 per cent of the whole extract; second, that while complete extraction of the residue from half the extract is possible, and is desirable in certain cases, it is a much more difficult and time-consuming procedure than complete extraction of the residue from 10 cc. It does not follow at all that since the cholesterol from a small residue may be extracted by a few minutes' boiling with chloroform the larger residue can be extracted by a similar short treatment. It is evident that failure to get a good extraction is probably an important cause of the inconsistent results, from the fact that lower values are obtained from Solution B which contains 50 per cent more cholesterol than Solution A. Whether the extraction of the serum with alcohol-ether was also at fault is not apparent, but it is evident that it was not always so, for the values which he obtained from the alcohol-ether extract saponified and determined in his own way are consistent and in fair agreement (when increased by 10 per cent to cover the error in aliquot) with those obtained by the various other methods. Thus for Solution A the reported value is 173 mg., and for Solution B 270 mg. These values increased 10 per cent would be 190 and 297 mg., which compare well with the values of 185 and 285 mg. obtained by the other methods. It is perhaps significant that these values obtained by his own method, with which he would presumably be most familiar, are the only consistent results obtained by the use of any part of my method.

My directions for the second part of the procedure, the pro-

duction of the color, call for the cholesterol from 10 cc. of alcohol-ether extract—ordinarily about 0.5 mg. (volumes to contain this amount are specified in later use of the method[4])—dissolved in 5 cc. of chloroform and compared with 0.5 mg. of cholesterol also in 5 cc. of chloroform; that is, the standard and test solutions are of about the same strength. They are also treated with the same amounts of reagents and are otherwise under parallel conditions—a plan of procedure which has been followed in all the methods for blood lipoids and which is believed to be the safe one for work in a new field. In Weston's version of my procedure a corresponding amount of serum extract is used for the test solution, but for the standard he used a solution containing 2.7 mg. of cholesterol in 10 cc. of chloroform—a standard approximately two and one-half times as strong as the test. For this strong solution the same concentration of acetic anhydride and sulfuric acid was employed as for the much weaker test solution. While the error introduced by comparing colored solutions so widely different in strength might not be great, it was improbable that either the quality or intensity of the color would be the same under these different conditions of treatment. That this is the case is shown by the following experiment. Chloroform extracts of blood cholesterol obtained by the Autenrieth and Funk method[5] (Solutions A, B, F, and G, below) were compared with standards prepared (*a*) according to my directions, standard 0.5 mg. of cholesterol in 5 cc. of chloroform treated with 2 cc. of acetic anhydride and 0.1 cc. of concentrated sulfuric acid; and (*b*) according to Weston's modification of my directions, standard 2.7 mg. of cholesterol in 10 cc. of chloroform treated with 4 cc. of acetic anhydride and 0.2 cc. of sulfuric acid. All were digested for 15 minutes in the dark at 22°C. The values, calculated in mg. per 100 cc. of blood, are shown in Table I.

The values obtained by Weston's modification are thus distinctly higher than those obtained by my directions. The colors were quite smoky in tint so that comparison was difficult. The high values may be in part due to the difference in tint, but probably more to the relatively low concentration of acetic

[4] Bloor, W. R., *J. Biol. Chem.*, 1916, xxv, 577.

[5] Autenrieth, W., and Funk, A., *Münch. med. Woch.*, 1913, lx, 1243.

TABLE I.

Solution.	By my directions.	By Weston's modification.
A	218	245
B	216	240
F	115	150
G	113	148

anhydride and sulfuric acid in Weston's standard which resulted in an incomplete development of the color.

Weston's experiment was repeated as follows:

Two samples of 50 cc. each of fresh beef plasma were taken and to one were added 5 cc. of ether containing 50 mg. of cholesterol, and to the other 5 cc. of ether alone (Weston did not add ether to his control serum). The two solutions were well shaken and after standing for about 2 hours with occasional shaking, samples were taken from them as follows.

a. Eight 3 cc. samples were taken from each and extracted with alcohol-ether according to directions in my method.[6] 10 cc. of the extracts were used for the determination.

b. Four 2 cc. samples were taken from each, digested with alkali, and extracted with chloroform according to the directions of Autenrieth and Funk.[5] The chloroform extracts after dehydration and filtering were made up to 100 cc. 15 cc. samples were used for the determinations and were measured into small beakers, evaporated to less than 5 cc., cooled, transferred to the 10 cc. cylinders, and made up to 5 cc. with the washings from the beakers.

c. Four 50 cc. samples of the alcohol-ether extracts from *a* were evaporated almost to dryness, then treated with alkali, and extracted with chloroform according to the directions of Autenrieth and Funk, using corresponding amounts of alkali and chloroform.

In all determinations the samples and standard were each contained in 5 cc. of chloroform and were treated with 2 cc. of acetic anhydride and 0.1 cc. of concentrated sulfuric acid in the dark at room temperature (20–22°C.) for 15 minutes. The Duboscq colorimeter was used throughout and there can be no question that it is more accurate and less subject to personal error than the Autenrieth-Königsberger instrument, certainly than the "*Hellige*" form which appears in this country. The Duboscq has also the advantage that the standard and test solutions are of about the same strength. It may be noted here that Autenrieth and Funk did not use a cholesterol standard, but instead used a permanent standard made of a mixture of metallic salts which was calibrated by the use of known cholesterol solutions, thus avoiding the difficulty mentioned

[6] Bloor, *J. Biol. Chem.*, 1916, xxiv, 227.

above. A permanent standard such as Autenrieth and Funk used has some advantages especially for use with the Autenrieth-Königsberger type of colorimeter, and as they do not give the composition of their solution, an attempt is being made to prepare a suitable solution.

The results of single consecutive determinations on these samples are given in Table II. The values are expressed in mg. per 100 cc. of blood.

TABLE II.

A. My procedure.		B. Autenrieth and Funk's procedure.	
		Directly on blood.	
Sample.	Value.	Sample.	Value.
Control plasma.		Control plasma.	
1	130	A	114
2	130	B	115
3	130	C	118
4	128	D	118
5	128	Plasma plus 1 mg. of cholesterol per 1 cc.	
6	129	E	218
7	129	F	216
8	129	G	218
		H	216
		On alcohol-ether extracts.	
Plasma plus 1 mg. of cholesterol per 1 cc.		Control.	
		1	119
1	227	Plasma plus cholesterol.	
2	229	1	219
3	229	2	214
4	230	3	219
5	229		
6	235		
7	235		
8	229		

These results show that the method will do all that has been claimed for it when used as originally directed. Satisfactory recovery of added cholesterol is obtained either by the use of

my method throughout or by the application of my procedure for color production to extracts obtained by the Autenrieth-Funk digestion either of plasma direct or of alcohol-ether extracts of it.

The alkali treatment either of plasma or of alcohol-ether extracts of it results in lower values.

The points enumerated below are sufficient to explain the low values reported by Weston but not the very high ones obtained by him in some cases. My method of cholesterol determination is based on the Liebermann-Burchard color reaction as used by Autenrieth and Funk, adapted for use with the alcohol-ether extraction of blood. The only departure from Autenrieth and Funk's directions (aside from the use of a cholesterol standard instead of a salt standard) is that the color production is carried out at room temperature instead of at about 32°C. (obtained by setting the color tubes in a beaker of water at 32–35°—in which the temperature may be 3° or 4° lower by the end of the 15 minutes). The change to room temperature was made as the result of experiments which showed that slightly higher results were obtained at the lower temperature and that the solutions were free from a yellowish tint which developed at the higher temperature. In the published directions no definite temperature was specified and as this seemed to be a possible source of error, temperature conditions were sought other than those in which the reaction was ordinarily carried out. Ordinarily the cylinders in which the color was produced were placed in the cupboard of a table in the middle of the laboratory where the temperature was at least as constant as that of the laboratory which is kept at 20–23°C. for all but the hottest months of the year. On the other hand, the temperature in a partly enclosed window-seat of a north window in the laboratory was found to be 12°C. A series of samples were therefore allowed to digest 15 minutes in a dark box there. The values obtained for blood cholesterol were very high, almost double those obtained at 21°C., and were quite irregular. The color in the standard cholesterol cylinder was obviously only little developed while that in the blood cholesterol cylinder was well developed. These results indicated that the standard cholesterol did not react at the same rate as the blood cholesterol, and other experiments to test this

point were carried out as follows. Solution A was a chloroform extract of residues from evaporation of mixed alcohol-ether extracts of human blood. Solution B was a standard cholesterol (Kahlbaum) solution in chloroform containing 0.5 mg. of cholesterol in 5 cc. Pairs of samples consisting of one each of these solutions were digested at temperatures of 10–34° as follows.

5 cc. of each solution were measured into a 10 cc. flask and 2 cc. of acetic anhydride added. The solution was adjusted to the required temperature by shaking in water at that temperature, 0.1 cc. of concentrated sulfuric acid added, the temperature again quickly adjusted, and the little flasks were placed in water in a beaker thermostat at the desired temperature, covered, and set in the dark for 15 minutes. At the end of that time the reaction was stopped by chilling the solutions in ice water and comparisons were made at once with the standard solution.

As a standard for each of the determinations a 5 cc. sample of the standard cholesterol was digested at 22° in the same way as the test samples, then chilled before use. The results of a typical series are given in Table III.

TABLE III.

Temperature.	Solution A (blood cholesterol).	Tint* of color.	Solution B (Kahlbaum's cholesterol).	Tint* of color.
°C.	mg.		mg.	
10	0.48±**	Blue.	Trace.	
14	0.62	Bluish.	0.21±**	Blue.
18	0.62	"	0.30±**	"
22	0.68	Matched.	0.50	Matched.
26	0.62	Faint yellowish.	0.52	Faint yellowish.
30	0.60±**	Yellow.	0.48	Yellowish.
34	0.58±**	Deep yellow.	0.40±**	Yellow.

* As compared with the standard.

** These values are approximate because of the difficulty of comparing colors of different tints.

Extracts from other blood samples showed some variations from the above, but in general the results were the same and indicate that the blood cholesterol behaves quite differently from the standard cholesterol. It reacts more readily with the re-

agents, comes to its maximum sooner, and begins to fade sooner than the standard (gall-stone?) cholesterol. Whether the blood cholesterol is wholly or in part a different substance from the standard or whether the difference in speed of reaction is due to an impurity in the blood extracts which accelerates the reaction is not clear, but in view of these differences in rate a further study of the Liebermann-Burchard reaction in its application to blood cholesterol is desirable and is now being undertaken in this laboratory.

The results obtained above indicate that values obtained at 22° are more nearly correct than those obtained at either higher or lower temperatures. At lower temperatures the standard color has not fully developed while at the higher temperatures the blood cholesterol color has passed its maximum and has begun to fade.

The experiments recorded in Table III throw an interesting light on the question of tints of color in blood cholesterol determinations. Mueller[2] and Weston[1] have both reported yellowish or brownish tints in the colors obtained from extracts made by my method. As noted above, the color, whether of blood cholesterol or of the standard cholesterol, passes through the stages of blue-green, green, yellow-green, and finally yellow, and whether the standard solution has a bluish or yellowish tint as compared with the test solution depends on the relative stage of development of the two. There is, of course, a considerable personal factor in judging tints of color, but in the course of a great number of blood cholesterol determinations carried out by my method at room temperature (22°) I have found very few in which the difference of tint was sufficient to make any difficulty in reading. In some cases, most frequently in extracts of whole blood, the alcohol-ether extract was brown or brownish green, and it was found that this color carried through into the chloroform. To get rid of this color Mueller's[2] directions have been found useful. After evaporating the alcohol-ether extract and extracting the residue with chloroform (using about 15 cc.), the chloroform is washed with water, dried with anhydrous sodium sulfate, evaporated to small volume, and the color produced in the cylinders as usual. The brownish color is soluble in water and is almost entirely removed by this treatment. A simpler way of

accomplishing the same result (due to Dr. W. E. McEllroy of this laboratory) is to shake the alcohol-ether extract with about 5 gm. of the anhydrous sulfate. Occasionally a sample is obtained (I have had three) in which the alcohol-ether extract is colorless but which develops a brown color in the chloroform on treatment with acetic anhydride and sulfuric acid. Washing or treatment with sodium sulfate was less effective in these cases. Some form of alkali treatment was necessary either with sodium ethylate, as was originally directed,[7] or with strong watery alkali and extraction, as in Autenrieth and Funk's method.

SUMMARY.

Results reported by Weston in the use of my method for the determination of cholesterol in blood are discussed and probable reasons for their inconsistency noted.

Differences in behavior of blood cholesterol and standard (Kahlbaum's) cholesterol are found.

A temperature of 22°C. has been found most suitable for the color production.

Causes of "off color" are discussed and suggestions offered for their correction.

[7] Bloor, *J. Biol. Chem.*, 1915, xxiii, 317.

A NOTE ON THE DIURNAL VARIATIONS IN CREATINE EXCRETION.

By W. DENIS.

With the Assistance of Anna S. Minot.

(From the Chemical Laboratory of the Massachusetts General Hospital, and the Biochemical Laboratory of the Harvard Medical School, Boston.)

(Received for publication, January 31, 1917.)

A short time ago during the course of a series of observations on the urine of a woman suffering from Graves' disease, we made the discovery that while the day urine of this patient contained considerable quantities of creatine, the urine passed during the night was practically creatine-free.

This observation has been followed by creatine determinations in the day and night urines of a number of hospital patients, with the result that in every case examined it has been found that the excretion of creatine is in a large measure confined to the hours between 7 a.m. and 7 p.m. The results of our observations are given in the tables.

The experimental subjects were all patients at the Massachusetts General Hospital. They were kept in bed during the period of observation and were fed during this period and 2 days previously a liberal diet free from meat, fish, peas, or beans.

The creatinine and creatine determinations were made by Folin's[1] micro methods. Picric acid carefully purified by the method described by Folin and Doisy[2] was used throughout the work.

In Table I are presented the results obtained by collecting the urine in 12 hour periods from 7 a.m. to 7 p.m. and from 7 p.m. to 7 a.m.

As will be seen, the quantity of creatine excreted during the night is but a fraction of that obtained in the day urine, while

[1] Folin, O., *J. Biol. Chem.*, 1914, xvii, 472.

[2] Folin, O., and Doisy, E. A., *J. Biol. Chem.*, 1916–17, xxviii, 349.

TABLE I.

No.	Sex.	Age.	Diagnosis.	Day. 7 a. m.—7 p. m.			Night. 7 p. m.—7 a. m.		
				Volume.	Preformed creatinine.	Creatine.	Volume.	Preformed creatinine.	Creatine.
		yrs.		*cc.*	*gm.*	*gm.*	*cc.*	*gm.*	*gm.*
1	♀	24	Graves' disease, mild case	560	0.32	0.15	500	0.30	0.02
2	♀	27	Graves' disease, mild case	1,500	0.32	0.21	820	0.37	0
3	♀	32	Graves' disease	1,100	0.37	0.26	505	0.31	0.08
4			Same patient	900	0.36	0.23	610	0.33	0.03
5	♂	65	Cirrhosis of liver, ascites	905	0.67	0.19	640	0.60	0
6	♂	44	Pneumonia, temperature 100–100.4° F.	650	0.71	0.23	660	0.70	0.02
7	♀	19	Graves' disease, very severe case	610	0.30	0.26	700	0.30	0.06
8	♂	10		860	0.18	0.16	665	0.17	0.03
9	♂	10	Nephritis	860	0.28	0.07	400	0.25	0.01

in some cases creatine is entirely absent from the night urine, while present in considerable quantities in that passed during the day.

In order to study to greater advantage the effect, if any, produced by the ingestion of food on the creatine output, we have

TABLE II.

Time.	Volume.	Total nitrogen.	Preformed creatinine.	Creatine.	Remarks.
	cc.	*mg.*	*mg.*	*mg.*	
7– 9 a.m.	90	810	51	20	Female, 24 yrs. old, weight 54.5 kg. Graves' disease.
9–11 "	120	1,010	52	32	Breakfast at 8.
11 a.m.–1 p.m.	200	925	50	10	Dinner at 12.
1–3 p.m.	175	1,100	60	44	
3–5 "	160	960	52	20	
5–7 "	210	1,186	54	32	Supper at 5.
7 p.m.–7 a.m.	440	2,422	290	30	

Total creatine excretion for the day (12 hrs.) 158 mg.
" " " " " night (12 ") 30 "

made 2 hour collections from 7 a.m. to 7 p.m. in five cases. The results of these observations are presented in Tables II to VI inclusive.

After the above work had been completed our attention was called to a paper by Powis and Raper[3] which we had overlooked. These investigators studied the diurnal and nocturnal variations in the

TABLE III.

Time.	Volume.	Total nitrogen.	Preformed creatinine.	Creatine.	Remarks.
	cc.	*mg.*	*mg.*	*mg.*	
7- 9 a.m.	156	672	56	70	Female, 20 yrs. old, weight 50 kg. Graves' disease, severe case.
9-11 "	210	900	52	102	Breakfast at 8.15.
11 a.m.-1 p.m.	320	1,185	60	84	Dinner at 12.20.
1-3 p.m.	275	1,100	60	42	
3-5 "	220	762	56	44	
5-7 "	190	505	55	52	Supper at 5.15.
7 p.m.-7 a.m.	430		360	60	

Total creatine excretion for the day (12 hrs.) 394 mg.
" " " " " night (12 ") 60 "

TABLE IV.

Time.	Volume.	Preformed creatinine.	Creatine.	Remarks.
	cc.	*mg.*	*mg.*	
7-11 a.m.	276	130	80	Female, 32 yrs. old, weight 68.1 kg. Graves' disease.
11 a.m.-1 p.m.	225	55	42	Breakfast at 7.45.
1-3 p.m.	475	70	80	Dinner at 12.20.
3-5 "	220	70	22	
5-7 "	370	70	41	Supper at 5.
7 p.m.-7 a.m.	700	420	110	

Total creatine excretion for the day (12 hrs.) 265 mg.
" " " " " night (12 ") 110 "

[3] Powis, F., and Raper, H. S., *Biochem. J.*, 1916, x, 363.

TABLE V.

Time.	Volume.	Total nitrogen.	Preformed creatinine.	Creatine.	Remarks.
	cc.	*mg.*	*mg.*	*mg.*	
7– 9 a.m.	150	422	30	20	Boy, 10 yrs. old, convalescent after amputation of leg.
9–11 "	170	530	34	41	Breakfast at 8.15.
11 a.m.–1 p.m.	110	490	29	18	Dinner at 12.30.
1–5 p.m.	415	1,330	65	39	
5–7 "	122	692	29	10	Supper at 5.
7 p.m.–7 a.m.	400	2,925	173	28	

Total creatine excretion for the day (12 hrs.) 128 mg.
" " " " " night (12 ") 28 "

TABLE VI.

Time.	Volume.	Preformed creatinine.	Creatine.	Remarks.
	cc.	*mg.*	*mg.*	
7– 9 a.m.	100	30	20	Boy, 12 yrs. old, weight 31.8 kg. Convalescent from nephritis.
9–11 "	150	33	10	Breakfast at 8.10.
11 a.m.–1 p.m.	210	29	11	Dinner at 12.30.
1–5 p.m.	410	70	41	Supper at 5.
5–7 "	100	27	15	
7 p.m.–7 a.m.	250	180	0	

Total creatine excretion for the day (12 hrs.) 97 mg.
" " " " " night (12 ") 0 "

excretion of creatine by a normal 3 year old child. They found that in this subject the rate of creatine excretion was highest during the morning, decreased somewhat in the later afternoon, and was very small during the night. These variations in creatine excretion are, according to Powis and Raper, not associated with the ingestion of food, but are due to the fact that the body when at rest has greater power to utilize creatine than when the muscles are in action.

As will be seen from our results the excretion of creatine by

persons on a strictly creatine-free diet varies from hour to hour; in most of the cases examined it attained a maximum in about 2 hours after the substantial meal of the day. Thus the subject used in Table I ate but little breakfast (a small dish of oatmeal, one slice of toast, and one glass of milk), but partook of a substantial dinner at noon (tomato purée, potato, two eggs, bread, milk, ice-cream); it will be noted that in her case the creatine excretion was not great during the morning hours, but attained a maximum between 1 and 3 o'clock in the afternoon. On the other hand the subject used in Table IV ate large quantities of food both at breakfast and dinner and shows a corresponding rise in creatine excretion after both of these meals.

It is also to be noted that on the whole the rise in nitrogen excretion following the ingestion of protein lags behind the curve of increased creatine excretion apparent after each meal.

Our results therefore do not confirm the finding of Powis and Raper as regards the lack of influence of food assimilation on creatine excretion. The source of urinary creatine, in spite of scores of investigations directed towards its elucidation, still remains obscure. The hypothesis that the creatine of the urine arises from the creatine of muscle is an inviting theory but one which as yet lacks indisputable proof. The acceptance of such a theory would place creatine among the metabolism products of endogenous origin. Our results make it seem highly probable, however, that creatine is of purely exogenous origin, and that its excretion is directly dependent on the intake of food.

DIRECT DETERMINATIONS OF PERMEABILITY.

By R. P. WODEHOUSE.

(Contributions from the Bermuda Biological Station for Research, No. 60, and from the Laboratory of Plant Physiology, Harvard University, Cambridge.)

(Received for publication, February 23, 1917.)

It is desirable to determine directly the penetration of substances into the living cell, but in most cases this is difficult or impossible. In order to make such direct determinations it is necessary to compare the composition of the cell sap with the composition of the liquid in which the cell is immersed. In the case of tissues this is impossible, because in crushing the cells to obtain the cell sap the latter is contaminated by admixtures of material from the intercellular spaces as well as altered by chemical action and adsorption. It is therefore necessary to employ individual cells and to extract their cell sap without opportunity for contamination or change.

Most cells are too small to yield satisfactory results by this method. The writer, however, has been fortunate in having an opportunity to experiment with the marine alga *Valonia*, which has individual cells so large that it is easy to get from a single cell enough sap upon which to make chemical tests. Cells of the average size yield from 1 to 2 cc. of sap, and it is not unusual to obtain cells which yield as much as 5 cc.

The cells were removed from the sea water, rinsed in distilled water, dried with filter paper, and then punctured with a needle. On pressing the cell wall the sap squirted out and was collected. This sap is contained in the large central vacuole of the cell (or cœnocyte).

Upon examination the cell sap was found to differ, in most cases, materially from sea water. The sap was taken from a few plants and tested qualitatively for the presence of some of the salts of sea water.

As for the metal ions of sea water, sodium could easily be demonstrated within the cell by allowing some of the sap to evaporate on a watch-glass when crystals of sodium chloride were seen to be formed. The presence of calcium was easily demonstrated. When a saturated solution of ammonium oxalate was added to the cell sap a white precipitate was produced which was soluble in hydrochloric acid but insoluble in acetic acid. This precipitate was washed in acetic acid and water and when dissolved in a small amount of hydrochloric acid gave an unmistakable flame test for calcium.

A similar condition, however, was not found to hold for magnesium. In sea water this can easily be demonstrated by precipitating with any of the fixed alkali hydroxides (*e.g.*, NaOH) forming $Mg(OH)_2$, which is white, gelatinous, and insoluble in excess of the reagent but readily soluble in the presence of ammonium salts, and from such solution can readily be precipitated by the alkali phosphates (*e.g.*, Na_2HPO_4) in the form of magnesium ammonium phosphate. When this method was tried on the cell sap, however, only the merest trace of a precipitate was obtained by the addition of NaOH and, though this disappeared upon the addition of ammonium sulfate, the addition of disodium phosphate to the solution would not cause the precipitation of any magnesium ammonium phosphate. This shows that, though magnesium is probably present within the cell, it does not exist in nearly as high a concentration as in sea water.

Exactly the opposite obtains in the case of potassium. In sea water the concentration of potassium is so low that no precipitate was produced by the addition of picric acid, and the dried residue gave only a doubtful flame test. With the *Valonia* sap, however, the dried residue showed an abundance of potassium by the flame test; when picric acid was added to the sap long acicular crystals settled out, and when these were dried and touched with a hot needle they detonated, giving an unmistakable potassium-colored flash, showing that these crystals, formed by the addition of picric acid, were potassium picrate. From this it is plain that there is a considerably higher concentration of potassium within the cell than without.

The *Valonia* sap gives an abundant precipitate with chloroplatinic acid. The solution was heated to complete the reaction

and when allowed to cool, the precipitate formed at room temperature and could be only slightly increased in amount by cooling to zero. Upon examination with the microscope this precipitate was found to consist mostly of octahedral crystals, the rest being made up of crystals of other forms but obviously of the same material because similar crystal forms were found in the precipitate in the control test with chemically pure potassium oxalate. This test seems to me to be conclusive for the presence of potassium. Its abundance in the sap is shown by a control test done on the Bermuda sea water. Keeping everything exactly the same it was impossible to get the potassium chloroplatinate crystals to form even by cooling down to zero. Small crystals could be caused to form only by evaporating the mixture nearly to dryness and adding several volumes of absolute alcohol. The conclusion arrived at from the picric acid test is thus confirmed.

The proportions of the four kations Na, Ca, Mg, and K, could not be determined quantitatively on account of the limited amount of material available at the time when the work was done. This is very important from the viewpoint of antagonism and the author hopes to make it the subject of further investigation.

When the anions of sea water were tested for the presence of chlorides they were indicated in all the tests made by the copious precipitate produced by the sap with silver nitrate. Barium chloride, however, failed to give any precipitate with the sap, thus showing the absence of sulfates. Nevertheless, barium chloride gave an abundant precipitate with the sea water from which the plants had been taken. Occasionally a cell was found which showed the presence of sulfate in the cell sap by the barium chloride test. Upon further investigation it was found that cells which were obviously dead always showed the presence of sulfate and that those which appeared to be in an unhealthy condition frequently gave a positive test. The cells which seemed to be healthy rarely gave any precipitate at all with barium chloride. Accordingly the following series of tests was made to determine if the presence of sulfate was associated with death or injury of the cell. On holding the cells up to the light the healthy ones were seen to have a uniform green color

over the entire surface, while cells which are described below as in poor condition had a mottled appearance, or the protoplasm appeared to be slightly shrunken, or else the cells, when held between the thumb and finger, could be felt to lack the normal turgidity.

In each case the description of the cell was written down before the test was made, in order to avoid subjective error.

No.	Description of cell.	$BaCl_2$ test.
1	Largest cell found (over 1 inch long).	No ppt.
2	Large, dead; protoplasm dead and shrunken.	Ppt.
3	Almost as large as No. 1; in good condition; old.	No ppt.
4	Small, average size, apparently healthy.	"
5	" " " " "	"
6	" " " " "	"
7	" " " " "	"
8	Large, but healthy.	"
9	Large; not in good condition but apparently still alive.	Ppt.
10	Appears to be in poor condition, not quite turgid but apparently still alive.	"
11	Much like No. 10.	No ppt.
12	Average cell in good condition.	"
13	" " " " "	"
14	Large and old but in good condition.	"
15	Small cell apparently healthy.	"
16	" " " "	Ppt.
17	Average cell in good condition.	No ppt.
18	An old cell but apparently healthy.	"
19	Normal, average size.	"

Besides these cells, which were taken as nearly as possible at random, thirty-six other cells were chosen as being undoubtedly healthy, and of these only one showed the presence of sulfate. From experience it is possible, by inspection, to be almost always, though never absolutely, certain which cells are going to show the presence of sulfate.

From this it seems evident that sap of live and healthy *Valonia* cells does not contain free sulfate in large enough quantities to give a visible precipitate with barium chloride. It is also equally certain that when the cell dies sulfate enters, and it follows from this that the presence of sulfate in the cell indicates injury or death, even though no other manifestations are visible.

In order to preclude the possibility that sulfate was present, but in some chemical or physical union which prevented it from reacting with barium chloride, *Valonia* sap which had been shown to give a negative test with this reagent was boiled with nitric acid of several different concentrations, in order to break up any such possible union, and tested again. In each case, however, the test for sulfate was negative.

In order to test the matter still further, some of the sap was incinerated in a porcelain crucible. Upon incineration it showed the presence of a great deal of organic material, which left a deposit of carbon, but upon being boiled with HCl and incinerated again this was removed and the residue was completely soluble in water. The sea water, on the other hand, showed no organic material and proved to be largely insoluble in water after the same treatment. The soluble part, however, gave a copious white precipitate with barium chloride, while the *Valonia* sap after incineration with HCl gave just a trace, scarcely enough to be visible.

The conclusion from the above experiment is that there is little or no sulfate within the vacuole of the normal *Valonia* cell. Consequently, since there is an abundance of sulfate in the surrounding sea water, the semipermeable protoplasmic membrane must possess a selective permeability which renders it impermeable to sulfate as long as it is alive. This, however, is lost immediately upon death.

It is of course possible that sulfate may penetrate the outer plasma membrane into the protoplasm without being able to penetrate the vacuole membrane into the vacuole, but upon this point the experiment throws no light.

In order to test for permeability to nitrates, some cells were placed in sea water to which enough potassium nitrate had been added to give a good nitron test. After 24 hours the cells were still alive (they remained alive for several days when replaced in sea water) and showed the presence of nitrate within by the nitron test. The same was true of sodium nitrate. But it appeared upon examination that the control cells in normal sea water also showed the presence of nitrates by the nitron test. Sea water did not show the presence of nitrate by the nitron test. It is therefore evident that the nitrates within the cell must have

penetrated through the plasma membrane, but this may have taken place wholly or in part before the nitrates were added to the sea water.

The mechanism by which nitrates and potassium are stored within the cell so as to remain at a higher concentration than in the surrounding sea water deserves further study.

It is of interest that the cell wall acts as an osmotic membrane but possesses no selective permeability. Dead cells are often turgid even though the protoplasm has shrunken away from the cell wall. These cells when put into a hypertonic salt solution almost immediately become flaccid, and when replaced in sea water or in fresh water they again become turgid. However, if dead cells are placed in water containing nitrates, nitrate can be demonstrated within them shortly afterwards. If living cells are killed and then replaced in sea water, sulfate can soon be shown to be present inside. This shows that salts can pass more or less freely through the cell wall and yet it offers sufficient obstruction to enable it to act as an osmotic membrane.

The writer takes pleasure in expressing his thanks to Professor E. L. Mark for the use of the facilities of the Bermuda Biological Station, where this investigation was made, to Professor W. J. V. Osterhout for his interest in the problem and many helpful suggestions, and to an unknown friend who assisted in bearing the expenses of the Bermuda trip.

THE MICRO-TITRATION OF AMMONIA, WITH SOME OBSERVATIONS ON NORMAL HUMAN BLOOD.

By GEORGE D. BARNETT.

(From the Laboratory of the Medical Division of the Stanford University Medical School, San Francisco.)

(Received for publication, February 3, 1917.)

In connection with the study of the ammonia poisoning which occurs in rabbits following the administration of large doses of urea, the problem of determining ammonia in small quantities of body fluids has recently come up in this laboratory, and certain refinements in the micro-titration of ammonia have been introduced. Our procedure differs from the usual aeration-titration method only in the use of an accurate microburette, 0.005 N alkali, and in a few details of titration, but it seems to offer certain advantages where the total quantity of ammonia available is very small, as in blood and tissue ammonia determinations, and in serial determinations of blood urea in small laboratory animals.

Method.

10 cc. of the solution to be investigated (containing less than 0.75 mg. of ammonia per 100 cc.) are placed in a large test-tube (22 x 380 mm.) and 10 cc. of saturated potassium carbonate solution added. The trace of ammonia present in the potassium carbonate solution should be removed by prolonged aeration of the stock bottle. Even with test-tubes of this depth a very small amount of alkaline "spray" will pass over into the absorption tube, but this can be prevented by interposing a short tube containing a loose plug of cotton, as suggested by Folin.[1] For absorption tubes we use still larger test-tubes (30 x 410 mm.) containing an accurately measured volume (less than 0.5 cc.) of 0.01 N hydrochloric acid in about 15 cc. of distilled water. This

[1] Folin, O., *Z. physiol. Chem.*, 1902–03, xxxvii, 161.

acid is measured from an automatic capillary pipette connected with a small stock bottle. If kept in seasoned glass the deterioration of this weak acid (and also of the alkali) is inappreciable over a period of a few weeks. The advantage in the use of deep absorption tubes is the entire absence of loss by spattering, even with vigorous aeration. An aeration rate of 5 liters per minute for 20 minutes is satisfactory. We have found it of advantage in maintaining a steady air-current to use a simple water bottle manometer calibrated in liters per minute by the method of Kober and Graves,[2] and connected to the distal end of the system which is being aerated.

If the absorption tubes are not of Jena or some "non-soluble" glass, a slight correction must be made for the alkali dissolved during the aeration. This correction is readily determined by passing the same air current through a sample of acid, and is constant for a given technique. In our tubes, and with the technique here given it amounts to 0.01 cc. of 0.005 N acid.

Titration of the excess acid is carried out in the absorption tube without removal of the aeration tube, a gentle current of air being passed through the acid for the purpose of stirring. 0.005 N alkali is run in from a 1 cc. microburette, made from a 1 cc. pipette graduated in hundredths and accurately calibrated. The tip of the burette is drawn out to deliver a drop of 0.01 cc. or less. Using methyl red as an indicator (four drops of a saturated solution in 70 per cent alcohol) a sharp end-point is readily obtained with half a drop (0.005 cc.) of the 0.005 N alkali. A fixed end-point is used for comparison, and sharper results are obtained if the color of the end-point is chosen where the pink color of the indicator just begins to show traces of yellow. For a fixed end-point we use a sodium acetate-acetic acid mixture. It is also of extreme importance to avoid the use of water containing carbon dioxide. Water from our laboratory Stokes still cannot be used at all unless boiled or redistilled over lime.

When the end-point is neared the stirring tube is removed, being carefully washed down by means of water from a bulb wash bottle; not from a bottle blown by mouth, on account of the carbon dioxide, nor from a shelf bottle, on account of

[2] Kober, P. A., and Graves, S. A., *J. Am. Chem. Soc.*, 1913, xxxv, 1594.

the alkali of the rubber tubing. The inside of the titration tube is then washed down, the volume made up to 25 cc., or any convenient small end-volume, as long as all samples are the same, and the titration completed, adding the alkali in successive amounts of 0.005 cc. and shaking or stirring gently before comparison with the fixed end-point.

Ten consecutive analyses of a solution of ammonium sulfate containing 0.12 mg. of NH_3 per 100 cc. gave results as follows:

1.	0.12	6.	0.12
2.	0.12	7.	0.11
3.	0.12	8.	0.12
4.	0.12	9.	0.11
5.	0.11	10.	0.12

We have applied the method to the determination of ammonia in oxalated human blood in a few normal cases. Since Medwedew[3] in 1911 called attention to the rapid formation of ammonia in shed blood it has been recognized that the figures for blood ammonia given by earlier investigators are much too high. Probably none of the results represent the actual preformed ammonia present in the circulating blood, but are largely merely a measure of the extent to which the labile ammonia-yielding bodies of the blood have been disintegrated. The results least open to criticism in this regard are those of Rohde,[4] who with the vividiffusion method found in the dialysate in equilibrium with dog blood 0.18 mg. of ammonia nitrogen per 100 cc. (= 0.19 mg. of NH_3).

As far as human blood is concerned, no means of overcoming the difficulty has been devised, and the best we can do is to minimize the error from the ammonia increase by beginning the aeration as soon as possible after the blood is drawn, and completing the analysis as rapidly as possible. The aeration methods of Folin have greatly facilitated such determinations. The only figures we have found for normal human blood published since the importance of rapid methods has been realized are those of Gettler and Baker,[5] who in a series of thirty cases, using the

[3] Medwedew, A., *Z. physiol. Chem.*, 1911, lxxii, 410.
[4] Rohde, A., *J. Biol. Chem.*, 1915, xxi, 325.
[5] Gettler, A. O., and Baker, W., *J. Biol. Chem.*, 1916, xxv, 211.

method of Folin and Denis,[6] found 0.4 to 0.75 mg. of ammonia nitrogen per 100 cc. (= 0.42 to 0.79 mg. of NH_3). It will be noted that these figures are about ten times those given here. Our aeration has been begun within 1 to 2 minutes after drawing the blood. In order to emphasize the importance of this, figures obtained from aeration of samples of the same blood begun after 30 minutes are given. It will be seen that the ammonia obtained is two or three times that of the samples aerated immediately.

Ammonia Found in Oxalated Human Blood.

No.	Aeration begun within.	NH_3 per 100 cc.	Aeration begun within.	NH_3 per 100 cc.
	min.	*mg.*	*min.*	*mg.*
1	1	0.05		
2	2	0.00	30	0.07
3	2	0.04	30	0.15
4	1½	0.03	30	0.10
5	2½	0.04	30	0.08

SUMMARY.

1. Modifications of the aeration-titration method of determining ammonia are described, for use with small amounts of fluids of low ammonia content.

2. Figures are given for ammonia obtained from fresh oxalated blood in five normal individuals.

[6] Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xi, 532.

STUDIES ON CHOLESTEROL.

III. THE INFLUENCE OF BILE DERIVATIVES IN BLOOR'S CHOLESTEROL DETERMINATION.*

PRELIMINARY REPORT.

BY GEORGINE LUDEN.

(From the Mayo Foundation for Medical Education and Research, Rochester, Minn.)

(Received for publication, February 23, 1917.)

The main object of the following communication was to determine whether bile derivatives were capable of giving the Liebermann reaction in the absence of cholesterol, and whether their color reaction could be destroyed by the use of sodium ethylate under conditions similar to those in Bloor's cholesterol determination.

The origin of the "brownish tint," well known to all who have made blood cholesterol determinations, and the relative merits of colorimetric and gravimetric methods for the determination of cholesterol have been repeatedly discussed (Bloor (2, 3, 4), Mueller, Weston). It will be remembered that Bloor, in the first publication of his new method (2), specially mentions the addition of sodium ethylate to the ether-alcohol extract of the blood before evaporation, and that he left out the sodium ethylate in his modification of this method (3): For brevity, I shall refer to Bloor's original method as Bloor I and to its modification as Bloor II. The comments of Mueller on the Bloor method for cholesterol determination and Bloor and Knudson's reply (4) to these comments both refer to the Bloor II method only. Mueller's conclusions were that the values obtained by the Bloor method were increased by "other ether-alcohol- and chloroform-soluble substances" that were not cholesterol; that oxycholesterol might be among these substances; but that our knowledge of oxycholesterol is too indefinite to warrant positive conclusions. Bloor and Knudson claimed to show by a new procedure that there were either no other substances in the blood plasma, or that they behaved like ordinary cholesterol when treated with digitonin.

* Study I, *J. Lab. and Clin. Med.*, 1916, i, 662; Study II, *J. Biol. Chem.*, 1916, xxvii, 273.

In connection with these statements a number of observations made by the writer since May, 1916, on a series of 748 parallel determinations with the Bloor I and II methods may be of interest. The series comprises 374 different blood samples including the blood of patients suffering from various diseases, the writer's blood in various experiments on nutrition, and the blood of goats used in an experiment that has already been published in part (11). It does not include the 400 blood samples mentioned in the latter publication.[1] It was found that in more than 100 specimens giving approximately normal cholesterol values, the difference between the Bloor I and II tests ranged from 0.050 to 0.070 mg., but in a group of samples derived from patients suffering from biliary disturbances, with and without icterus, it increased from 0.090 to 0.280 mg. The highest value with the Bloor II test was found in the blood of a highly icteric patient and the deduction seemed admissible that it might be due to the presence of bile derivatives which the Bloor I tests had eliminated by the use of sodium ethylate.

Bile acids do not give the Liebermann-Burchard reaction (6, 8) but it seemed possible that a combination of bile pigments and bile acids such as is often found in icteric blood, and usually in gall-stones, might create conditions favorable for the reaction. The residue of gall-stones from which the cholesterol had been thoroughly extracted (eleven successive extractions with boiling alcohol on the water bath) was therefore selected for investigation. This residue dissolved readily in chloroform with the exception of a black, dust-like sediment. The latter was collected on filter paper, dried at room temperature, and used as the starting point for the experiments. It was dissolved in 2 per cent ammonia water and shaken out with pure chloroform in a separatory funnel. The chloroform was tested for cholesterol and remained colorless; however, as a measure of precaution, the operation was repeated three times. The third amount of chloroform having been drawn off completely, the aqueous alkaline solution of the black sediment was acidified drop by drop with concentrated hydrochloric acid and again shaken out with fresh chloroform. The latter assumed a deep yellow tone, and on addition of the Liebermann reagents (2 cc. of acetic anhydride and 0.1 cc. of concentrated sulfuric acid to a 6 cc. portion of the chloroform solution) gave a vivid green reaction such as is found only in strong cholesterol solutions; *i.e.*, 1 mg. to 1 cc. of chloroform.

[1] Luden (11), p. 293, footnote 2.

The amount of black sediment dissolved in the ammonia water had been extremely small—only as much as could be held on the point of a small scalpel—since the entire yield of black sediment had not exceeded 150 mg. and it had been necessary to make a great number of experiments before the most suitable method of extraction had been found. An equally great number of experiments, in which sodium and potassium hydroxide had been used as solvents, had resulted in failure, showing that the relative alkalinity of the solvent was not without importance.

The color reaction obtained with the yellow chloroform extract of the bile derivatives must be considered a true Liebermann reaction since it was produced by identical proportions of the same reagents used for cholesterol tests.

The absence of cholesterol in the yellow chloroform extract may be deduced from: (*a*) the insolubility of the black sediment in chloroform; (*b*) its solubility in ammonia water; (*c*) the repeated extraction of the aqueous alkaline solution with pure chloroform, which gave no Liebermann reaction although the procedure was practically identical with that of Autenrieth; and (*d*) the method by which the yellow chloroform extract giving the Liebermann reaction had been obtained, a method similar to that used for the recovery of bilirubin (15), urobilin (16), and cholic acid (21). It had therefore been demonstrated that a positive Liebermann reaction may be obtained from a mixture of bile derivatives in chloroform solution in the definite absence of cholesterol.

Windaus (20) states that Liebermann's cholesterol reaction is characterized by a sequence of brilliant colors, vivid pink, blue, and dark green. This color reaction could be observed in all of these bile derivative solutions even when they contained as little as 0.020 mg. in 6 cc. of chloroform, but I have been unable to find any trace of the pink or the blue stage of the reaction in cholesterol solutions such as are commonly used for blood cholesterol determinations (0.4 mg. in 6 cc. or 0.5 mg. in 5 cc. of chloroform). Since only the green stage of the reaction can be seen in cholesterol solutions of the above concentration, it was decided to ascertain how great the concentration of cholesterol solutions would have to be, the usual proportions of the reagents being maintained, in order to show the color sequence described by Windaus. It was found that neither the pink nor the blue

stage could be observed in cholesterol solutions exceeding the concentration of standard tests from 10 to 400 times (Table I). Nor could the color sequence described by Windaus be made visible by using one-half or one-quarter of the amount of the reagents.

For brevity, the bile derivative solutions used in my experiments will be referred to as "pigment solutions" and the uniform solution with which the following tests were made as "Pigment 28 solution." The latter contained 0.2 mg. of bile derivatives in every 6 cc. of chloroform.

TABLE I.

*Color Reactions in Cholesterol Solutions.**

Test 1.—6 cc. of our stock solution, namely, 6 mg. of cholesterol in 6 cc. of chloroform; usual reagents added.

Reaction.—The colorless solution becomes dark green in a few seconds; no trace of the pink or the blue stage.

Test 2.—10 mg. of cholesterol in 6 cc. of chloroform; usual reagents added.

Reaction.—Identical with Test 1, except that the green color appears almost black.

Test 3.—15 mg. of cholesterol in 6 cc. of chloroform; usual reagents added.

Reaction.—The blue stage appears for a few seconds only and is blotted out by the density of the green stage. The solution appears black; its green tone can be recognized only in a very strong light.

Test 4.—200 mg. of cholesterol dissolved in 6 cc. of chloroform; usual reagents.

Reaction.—As the sulfuric acid is added, its progress towards the bottom of the test-tube is shown by a streak of purple-violet, but the next second the whole of the solution has turned black-green. Its green color can be recognized only near the top edge by shaking the test-tube.

---

* The standard cholesterol solution used in all our blood cholesterol determinations contains 0.4 mg. of cholesterol in 6 cc. of chloroform. It is made up from a stock solution containing 200 mg. of cholesterol (Merck) in 200 cc. of chloroform.

Both standard and stock solutions are sealed with paraffin and kept on ice when not in use, in order to prevent any evaporation of the chloroform; the latter would increase their concentration, thereby affecting the accuracy of the tests.

Standardized graduated pipettes are used in all our tests in preference to graduated cylinders, since the latter often vary slightly and give less accurate results in consequence.

Apart from the color sequence referred to above, the pigment solutions showed the following peculiarities, which distinguished them from pure cholesterol solutions: (*a*) Their reaction was much slower than that of cholesterol; (*b*) the green stage of the reaction was olive-green, as compared with the emerald-green of pure cholesterol; (*c*) the green stage persisted unchanged from three to four times as long as the cholesterol green in solutions of equal strength; and (*d*) colorimetric determinations showed that the color value of the green stage was only a little over one-half of the cholesterol green in solutions of equal concentration, although, owing to the difference in tone, it appeared to be greater in the test-tube. In reference to these peculiarities the term "bile-green reaction" in the Liebermann test is tentatively suggested for the color reaction of these cholesterol-free bile derivatives.

A series of observations and systematic tests proved that the usual cholesterol standard test (0.4 mg. of cholesterol in 6 cc. of chloroform) reached its maximum color value at room temperature (20–22°C.) in 5 to 6 minutes, and maintained it for approximately 30 minutes; that the test lost one-third of its color value in 80 minutes and became colorless, *i.e.*, pale yellow, in 6 to 7 hours. On the other hand, pigment solutions of identical concentration reached their maximum color value in 190 minutes and remained unchanged for at least 24 hours. A record was kept of the exact time at which the reagents were added to the tests and revealed the length of the duration of the pink, blue, and green stages of the reaction in the pigment solutions at a room temperature of 20–22°C. (Table II). In every instance freshly made "ripe" standard tests not more than 20 minutes old were used for comparison. The advantage of making cholesterol determinations at room temperature rather than at a temperature of 35–37°C. has been discussed (11). Previous observations on the rapid reaction found in many pathologic blood samples have been fully corroborated by subsequent findings.

TABLE II.

Color Reactions in Pigment Solutions.

2 cc. of acetic anhydride and 0.1 cc. of concentrated sulfuric acid added to each 6 cc. test. Room temperature 20–21°C.

Test 1.—1.2 mg. in 6 cc. of chloroform. Pigment 28 solution undiluted (three tests).

Reaction.—The test solution turns bright reddish pink immediately. The pink color lasts for 20 minutes. The test-tube is plunged in very hot water for a few seconds. The blue stage of the reaction appears, but has a dirty violet-blue tone. It is followed in 3 to 4 minutes by the green stage, intense olive-green, which seems to be as dark as, if not darker than, cholesterol green of equal strength in the test-tube.

Color Value.—After 30 minutes equal to 0.500 mg. cholesterol. (Standard cholesterol test* 0.400 mg.)

After 6 hours " " 0.666 " "
" 24 " " " 0.666 " "
" 48 " " " 0.666 " "
" 72 " faded to a dirty brown.

Test 2.—0.400 mg. in 6 cc. of chloroform (five tests).

Reaction.—The test solution turns bright vivid pink immediately. The pink color lasts for 5 to 7 minutes. Dirty violet tone for 3 to 4 minutes. Olive-green.

Color Value.—After 20 minutes equal to 0.180 mg. cholesterol. (Standard cholesterol test 0.400 mg.)

After 1 hour " " 0.200 " "
" 3 hours " " 0.200 " "
" 24 " " " 0.222 " "
" 48 " faded to a dirty brown. (Standard cholesterol test fades to dirty yellow in 6 hours.)

Test 3.—0.120 mg. in 6 cc. of chloroform (three tests).

Reaction.—Vivid pink for 5 minutes. Intermediate stage hard to define. Green in 10 minutes.

Color Value.—After 30 minutes equal to 0.060 mg. cholesterol. (Standard cholesterol test 0.400 mg.)

After 3 hours " " 0.056 " "
" 24 " faded to dirty yellow.

Test 4.—0.080 mg. in 6 cc. of chloroform (three tests).

Reaction.—Clear pale pink for 3 minutes. Intermediate color cannot be recognized. Clear but light olive-green in 6 to 8 minutes.

Color Value.—After 30 minutes equal to 0.050 mg. cholesterol. (Standard cholesterol test 0.400 mg.)

After 3 hours " " 0.040 " "
" 24 " faded to pale dirty yellow.

---

* A newly made "ripe" standard was used for every determination, and for every test the Duboscq colorimeter was set at 10.0 mm.

TABLE II—*Concluded.*

Test 5.—0.040 mg. in 6 cc. of chloroform (three tests). (Standard cholesterol test 0.400 mg.)

Reaction.—Clear pale pink; then pale olive-green which lasts for about 3 hours.

Color Value.—Cannot be determined as the test looks gray in the colorimeter when compared with the emerald-green standard, although its color is clear pale green in the test-tube.

Test 6.—0.020 mg. in 6 cc. of chloroform (three tests). (Standard cholesterol test 0.400 mg.)

Reaction.—Faint but distinct pink; then very pale olive-green lasting about 1 hour.

Color Value.—Cannot be determined, for the same reasons as in Test 5. The green color is also clearly visible in the test-tube.

In order to study the effects of sodium ethylate on the cholesterol-free bile derivatives under conditions parallel to those found in the Bloor method, the following experiments were made:

The chloroform of a portion of the Pigment 28 solution was evaporated and the orange-colored residue extracted with ether-alcohol in the manner described by Bloor for blood samples. One portion of the ether-alcohol extract was treated according to the Bloor I method, with sodium ethylate, and the other according to the Bloor II method, without sodium ethylate. Overheating was carefully avoided during evaporation. After the usual extraction of the residue with chloroform, the Bloor I test was colorless and remained colorless when the usual reagents were added, even when the test was warmed. The Bloor II chloroform, on the contrary, was deep yellow and on the addition of the reagents gave the brilliant bile-green reaction already described. The experiment was repeated four times and gave identical results in every instance. Parallel tests made with pure cholesterol solutions treated in exactly the same way gave positive Liebermann reactions both with the Bloor I and the Bloor II methods. A slight loss of color value occurred in the pigment as well as in the cholesterol control tests. In the latter it was so small that it may be considered merely technical; 0.233 and 0.572 mg. of cholesterol were recovered when 0.240 and 0.600 mg. had been used respectively. In the former the loss was somewhat greater, but the factors which caused it cannot

be considered at present. The experiments showed conclusively, however, that the color reaction of cholesterol-free bile derivatives can be destroyed by sodium ethylate, whereas that of true cholesterol is not similarly affected. It should be added that the bile derivatives were readily soluble in ether, alcohol, chloroform, and petroleum ether (14), but that a precipitate was formed whenever sodium ethylate was added to the chloroform solution, whereas in the other solvents precipitation was barely perceptible. The bile-green type of Liebermann reaction could be obtained also with the usual reagents from the opaque residue left in the beakers used for the Bloor I tests after chloroform extraction by dissolving the residue in acid chloroform. These findings seem to support the deduction that the formation of a chloroform-insoluble sodium salt of bile derivatives may account for the difference observed in the Bloor I and Bloor II samples, and that the potassium salts of these substances may be more readily soluble in chloroform, especially in the presence of water. The occurrence of the brownish color in some of the Autenrieth tests and Mueller's[2] observations might also be explained thereby.

Although these experiments showed that the color reaction of bile derivatives obtained from gall-stones can be destroyed by sodium ethylate, it remained to be proved that closely allied or identical bodies were responsible for the high values found in icteric blood by the Bloor II method. The isolation of the bile derivatives in icteric blood proved extremely difficult, probably owing to the relatively small quantities in which they are present. Thirty attempts to isolate them were unsuccessful. It is possible that these failures may be explained by the observations of Hoover and Blankenhorn on "dissociated jaundice" and that several of the samples used in my experiments contained only bile acids. However, there may be other reasons connected with the

[2] The brownish color does not appear in every sample treated by the Autenrieth method, and I am unable to agree with Mueller's statement that it is "invariably present" in Bloor II tests. On the contrary, one-third of our determinations gave a brilliant green reaction that could be matched easily with the standard solution. Tests made by the Bloor I method always gave a perfect match.

various stages of oxidation described by Lifschütz (6–10) but these cannot be discussed at present.[3]

At last a simple method was found by which the bile derivatives of icteric blood could be isolated: 6 cc. of icteric blood were extracted with ether-alcohol according to the Bloor method. The extract was partly evaporated at room temperature. The remaining portion of the solvent, in which cholesterol crystals could be observed microscopically, was poured off. A black crust-like ring which had formed where the liquid receded during evaporation was dissolved in 2 per cent ammonia water, and the aqueous alkaline solution filtered, acidified, and shaken out with chloroform. The chloroform assumed a yellow tone and gave the bile-green type of Liebermann reaction. The bile derivatives, in this specimen of icteric blood at least, must therefore have been closely allied, if not identical with the cholesterol-free bile derivatives obtained from gall-stones. The difference between the Bloor I and Bloor II tests of this sample had been 0.280 mg. in the colorimetric determination for blood cholesterol.

A test devised by Lifschütz[4] for the differentiation between oxycholesterol and oxidized cholic acid proved of great value in establishing the identity of the bile derivatives in question. This test will be referred to as Lifschütz' "differential test." It is based on the relative solubility of oxycholesterol and oxidized cholic acid in chloroform and glacial acetic acid, and should not be confounded with the oxycholesterol color reaction. Lifschütz states that the oxycholesterol color reaction can be obtained alike in glacial acetic solutions of pure cholesterol, pure cholic acid, and ordinary bile after oxidation with benzoyl peroxide and the subsequent addition of eight drops of concentrated sulfuric acid and one drop of ferric perchloride (2 per cent solution in glacial acetic acid) to every 1 cc. portion of the test. If an equal volume of chloroform is added to the glacial acetic solutions, the green color of the oxidized cholesterol will be found in its entirety in the upper layer, the bottom layer being practically

[3] Because of Lifschütz' observations on the decomposition of cholesterol to bile acids, and Schulze and Winterstein's studies on the influence of the light on cholesterol, my pigment solutions have always been kept in the dark.

[4] Lifschütz (9), p. 346.

colorless (pale yellow or brownish), whereas if the test is made with oxidized cholic acid the colors will be reversed, the upper stratum will remain colorless after separation, and the bottom (chloroform) layer will contain all the green color. When bile is used, both layers will be colored in direct proportion to the amount of either substance present in each layer; the quantity can then be determined only spectroscopically, by means of the characteristic spectra. In the absence of spectroscopic facilities, however, oxycholesterol and oxidized cholic acid can be differentiated accurately by means of the differential test when only one substance is present in the solution. Lifschütz adds that the proportions he recommends must be strictly observed in order to obtain the above results. These proportions have therefore been used in the following experiments.

The chloroform component of (*a*) a cholesterol solution, (*b*) a portion of the Pigment 28 solution, and (*c*) a solution containing the cholesterol-free bile derivatives of icteric blood, was removed by evaporation and the residues were dissolved in glacial acetic acid. The glacial acetic solutions were treated in the manner described by Lifschütz and the differential test was made by adding an equal volume of chloroform. After separation had taken place the green color of the oxidized cholesterol was found in the upper layer, while the bottom layer had a faint pinkish brown tinge. In the two tests made with the bile derivatives, on the contrary, the upper layers were colorless and the green color could be seen in its entirety in the lower (chloroform) layers. Whether the differential test was made at the red, the blue, the green, or the terminal dirty brown stage of the reaction, the color component of the oxidized cholesterol was found in the upper stratum, whereas that of the bile derivatives invariably settled in the bottom layer. Moreover, the green stage of the bile derivatives remained unchanged for days, while the oxycholesterol green changed to dirty brown in about 12 hours. The latter peculiarity alone, which is in accordance with the color persistency of the bile-green reaction in my pigment solutions, might suffice to distinguish the bile derivatives from oxycholesterol, and recalls Lifschütz' statement concerning the color reaction of oxidized cholic acid, that colors like spectra remain for weeks. Although these tests showed that the bile

derivatives contained no oxycholesterol, but substances that were closely allied to cholic acid, a slight difference could be observed between the derivatives from gall-stones and those from icteric blood. The latter needed the complete oxidation required by cholic acid, while the former gave the Lifschütz reaction after oxidation with benzoyl peroxide alone, without the addition of sulfuric acid and ferric perchloride. It will be remembered that the color sequence of the Lifschütz reaction is the reverse of that seen in bile pigments under the influence of oxidizing agents.[5] The gall-stone derivatives must therefore have been partially oxidized. It is hard to understand where this partial oxidation could have occurred as no oxidizing agents except the atmospheric oxygen during extraction of the cholesterol had been used until Lifschütz' differential test was made. The following explanation is tentatively suggested: As bilirubin, a hemoglobin derivative (Mathews), could be demonstrated in the mixture by Hammarsten's test, and as Lifschütz (10) was able to prove the strongly oxidizing properties of hemoglobin, it seems possible that some of the bile pigments present in the mixture may have played a part in the partial oxidation of the cholic acid which it contained. The bile derivatives from icteric blood exhibited the following peculiarity: Although the upper layer remained colorless in Lifschütz' differential test, the colored bottom layer was composed of two strata, the lower and narrower being dark green, the other bright yellow with a green tinge. This showed that the bile derivatives did not contain oxidized cholic acid alone, but another closely allied substance as well. The latter may have been the "rhizocholic acid" mentioned by Mathews[6] or the "resin acid" described by St. Minovici and Zenovici.

Further investigations concerning the solubility of the bile salts, spectroscopic determinations, and tests with Rosenheim's new reaction for oxycholesterol will be reported when completed.

SUMMARY.

1. In 748 parallel blood cholesterol determinations by Bloor's original method with sodium ethylate (Bloor I) and its modification without sodium ethylate (Bloor II), a constant difference

[5] Von Reinbold (15), p. 278.

[6] Mathews (12), p. 430.

was observed in the cholesterol values obtained, lower values being registered by the Bloor I method.

2. In normal blood a constant slight difference (0.050 to 0.070 mg.) occurs between the values obtained by the Bloor I and Bloor II methods; in icteric blood differences of 0.090 to 0.280 mg. occur.

3. Since the Liebermann reaction could be obtained with a mixture of cholesterol-free gall-stone derivatives, the difference appears to be due to a combination of bile pigments and bile acids present in the blood.

4. The Liebermann reaction of cholesterol-free gall-stone derivatives differs from that of cholesterol inasmuch as the pink stage of the reaction is distinctly visible in weak solutions of the former, although it cannot be seen in cholesterol solutions of equal or up to 400 times greater concentration.

5. The type of Liebermann reaction given by gall-stone derivatives can be obtained from the cholesterol-free residue of icteric blood.

6. This color reaction is destroyed by the use of sodium ethylate under conditions parallel to those found in the Bloor I method. The color reaction of cholesterol is not affected by the use of sodium ethylate.

7. That the Liebermann reaction of cholesterol-free bile derivatives is due, not to oxycholesterol, but to cholic acid and allied substances in the presence of bile pigments, could be proved by Lifschütz' differential test.

8. Cholesterol-free gall-stone derivatives require less vigorous oxidation than bile derivatives from icteric blood in order to give Lifschütz' differential test.

9. The oxidizing properties of hemoglobin having been demonstrated by Lifschütz, and bilirubin, a hemoglobin derivative, being demonstrable in the gall-stone derivatives, it is tentatively suggested that the bile pigments contained in the latter may have caused their partial oxidation.

10. The experiments suggest that parallel determinations with Bloor's original method and its modification may furnish valuable information concerning the chemical constituents of the blood in cases of biliary disturbance with and without icterus, which might be supplemented by the dialyzation method of Hoover and Blankenhorn.

BIBLIOGRAPHY.

1. Autenrieth, W., and Funk, A., Ueber kolorimetrische Bestimmungsmethoden: Die Bestimmung des Gesamtcholesterins im Blut und in Organen, *Münch. med. Woch.*, 1913, lx, 1243.
2. Bloor, W. R., Studies on blood fat. II. Fat absorption and the blood lipoids, *J. Biol. Chem.*, 1915, xxiii, 317.
3. Bloor, The determination of cholesterol in blood, *J. Biol. Chem.*, 1916, xxiv, 227.
4. Bloor, W. R., and Knudson, A., The separate determination of cholesterol and cholesterol esters in small amounts of blood, *J. Biol. Chem.*, 1916, xxvii, 107.
5. Hoover, C. F., and Blankenhorn, M. A., Dissociated jaundice, *Arch. Int. Med.*, 1916, xviii, 289.
6. Lifschütz, J., Die Oxydationsprodukte des Cholesterins in den tierischen Organen. III Mitteilung, *Z. physiol. Chem.*, 1908–09, lviii, 175.
7. Lifschütz, Die Oxydationsprodukte des Cholesterins in den tierischen Organen (Pfortader-Lebervene). V Mitteilung, *Biochem. Z.*, 1913, lii, 206.
8. Lifschütz, Der Abbau des Cholesterins in den tierischen Organen (Studie). VI Mitteilung. (Cholesterin-Gallensäuren), *Z. physiol. Chem.*, 1914, xci, 309.
9. Lifschütz, Die Abbauprodukte des Cholesterins in den tierischen Organen (Gallensäuren). VII Mitteilung, *Z. physiol. Chem.*, 1914, xcii, 383.
10. Lifschütz, Die Oxydation des Cholesterins durch das Blutgewebe. VIII Mitteilung, *Z. physiol. Chem.*, 1914–15, xciii, 209.
11. Luden, G., Observations on the changes in the cholesterol content of the blood of goats following cholesterol feeding alone, roentgen treatment alone, and cholesterol feeding combined with roentgen treatment and subsequent castration, *J. Biol. Chem.*, 1916, xxvii, 273.
12. Mathews, A. P., Physiological Chemistry, New York, 1915.
13. Minovici, S., and Zenovici-Eremie, T., Some new oxidation products of cholesterol, *Bull. sect. sci. acad. romaine*, 1915, iv, 194 (abstracted in *Chem. Abst.*, 1916, x, 606).
14. Mueller, J. H., A comparison of the results obtained by colorimetric and gravimetric determinations of cholesterol, *J. Biol. Chem.*, 1916, xxv, 549.
15. Von Reinbold, B., Urobilin, in Abderhalden, E., Biochem. Handlexikon, Berlin, 1911, vi, 278.
16. Von Reinbold, Urobilin, in Abderhalden, Biochem. Handlexikon, Berlin, 1911, vi, 288.
17. Rosenheim, M. C., A new colour reaction for "oxycholesterol," *Biochem. J.*, 1916, x, 176 (abstracted in *Chem. Abst.*, 1916, x, 3081).

18. Schulze, E., and Winterstein, E., Über das Verhalten des Cholesterins gegen das Licht, *Z. physiol. Chem.*, 1904–05, xliii, 316 (cited by Lifschütz).
19. Weston, P. G., Colorimetric methods for determining serum cholesterol, *J. Biol. Chem.*, 1916–17, xxviii, 383.
20. Windaus, A., Cholesterin, in Abderhalden, Biochem. Handlexikon, Berlin, 1911, iii, 270.
21. Windaus, Cholsäure (früher Cholalsäure), in Abderhalden, Biochem. Handlexikon, Berlin, 1911, iii, 315.

THE HOURLY ELIMINATION OF CERTAIN URINARY CONSTITUENTS DURING BRIEF FASTS.

By ISAAC NEUWIRTH.

(From the Department of Chemistry, Cornell University Medical College, New York City.)

(Received for publication, February 28, 1917.)

The data presented in the present paper form the first part of a contemplated study of the influence of various agents (chiefly foodstuffs) upon the elimination of certain urinary constituents, with special reference to uric acid. As originally planned the work was to be essentially a repetition of the recent work of Mendel and Stehle,[1] making use of the microcolorimetric method for uric acid determinations, instead of the Folin-Shaffer[2] method, which Mendel and Stehle employed. We believe that the Folin-Shaffer method is not well adapted to the study of *hourly* uric acid elimination,[3] especially where the output falls below 10 mg. or the volume of urine is large. As the work progressed, it became apparent that the hourly uric acid output during brief fasts is influenced by different factors, which for the most part we have been unable to account for satisfactorily. In order to find whether other urinary constituents were subject to parallel fluctuation with the uric acid, the work was extended to include creatinine and total nitrogen. An examination of the literature has failed to bring to light satisfactory data regarding this point.

The subject of the experiments is 22 years of age and weighs 55 kilos. His activity during the experiments consisted of laboratory routine. The analytical methods employed were the following: total nitrogen, Kjeldahl; creatinine, Folin's colorimetric

[1] Mendel, L. B., and Stehle, R. L., *J. Biol. Chem.*, 1915, xxii, 215.
[2] Folin, O., and Shaffer, P. A., *Z. physiol. Chem.*, 1901, xxxii, 552.
[3] Benedict, S. R., *The Harvey Lectures*, 1915–16, xi, 346.

method; uric acid, the Folin-Denis method as modified by Benedict and Hitchcock.[4]

The routine of the experiments was as follows. For 3 days before each experiment the diet was essentially free from purines. No food was eaten from 6 p.m. on the day preceding an experiment until after the experiment was finished. The urine was collected in hourly periods from 7 a.m. to 9 p.m., special care being taken to empty the bladder completely each time. The urines collected up to 4 p.m. were analyzed the same day, the later samples were preserved with toluene and analyzed the day following.

Table I, Experiments 1 and 2, records experiments in which the uric acid output only was determined and in which the quantity of water ingested was low but was not measured. The results corroborate the previous findings of Smetánka[5] and of Mendel and Stehle. There is a marked fall in the uric acid output during the morning hours, which is followed by a more gradual decline during the afternoon. An inspection of the tables will show, in agreement with the work of Mendel and Stehle, that there seems to be an unmistakable relationship between urinary volume and uric acid excretion, but only within certain limits. As a rule a decrease in urinary volume is accompanied by a decrease in uric acid, but numerous exceptions to this may be noted in the experiments, particularly in the early morning hours.

Experiment 3, Table I, records an experiment in which neither food nor water was ingested. The urine was collected in periods of approximately 3 hours. This experiment differed from the others reported in this paper in that a purine-containing food (chicken) was ingested about 5 o'clock in the afternoon preceding the experiment. The results illustrate the relation of uric acid to urinary volume, but do not appear to differ materially from those obtained in the other experiments.

[4] Benedict, S. R., and Hitchcock, E. H., *J. Biol. Chem.*, 1915, xx, 619. Dr. Benedict has modified the preparation of the Folin-Denis uric acid reagent as follows: Boil together 100 gm. of sodium tungstate, 30 cc. of 85 per cent phosphoric acid, 20 cc. of concentrated hydrochloric acid, and 750 cc. of water under a reflux condenser for 1½ hours. This modified solution shows less tendency to develop turbidity than does the original Folin-Denis reagent. Cool and make up to 1 liter.

[5] Smetánka, F., *Arch. ges. Physiol.*, 1911, cxxxviii, 217.

In Table II are recorded four experiments in which the intake of water was constant (100 cc. per hour), and in three of which the total nitrogen and creatinine have been determined for each hour. With a constant intake of a fairly liberal quantity of water the excretion of uric acid is somewhat more regular than in our first experiments (where the water intake was low but was not measured) or as compared with Experiments 8 and 9, where the water intake was low or irregular. The figures are of value as showing the variations to be expected in the same individual under apparently identical conditions. Particular attention is drawn to Experiment 5 which serves to illustrate a serious source of error in interpreting results upon the uric acid output. If, in Experiment 5, some material had been ingested at about 1 or 2 o'clock the inference might have been drawn that it increased the uric acid elimination, whereas the experiment really shows only the variation to be expected without ingestion of anything but a constant quantity of water throughout. It is probably safer, in interpreting uric acid results, to take as a basis a series of experiments such as is here given, rather than to rely upon the "control" period covering a few hours before the ingestion of the material to be studied.

A comparison of the figures for uric acid with the total nitrogen in Experiments 5, 6, and 7 will show that there is a remarkably close correspondence in the rise and fall of these two constituents. A given quantity of nitrogen in gm. does not correspond to a given weight of uric acid in the different experiments, but the relative rise and fall of the two are so nearly constant as to lead one to suspect that either may be regarded as a general index of kidney efficiency with respect to both. This point is one which we believe should be taken into account in any study of the effects of various agents upon uric acid formation. From our results it appears not improbable that when the kidney is called upon to do additional work in the excretion of one constituent it may respond by increased efficiency in respect to other constituents.

The relation between uric acid elimination and the creatinine output is not quite so definite as that between uric acid and the total nitrogen. In a general way the creatinine elimination also parallels the total nitrogen, but it will be seen that this relation is not so constant as for the uric acid.

In connection with the data on the creatinine elimination, it is of interest to note that the hourly output of this constituent during brief fasts is apparently not nearly so constant as Shaffer[6] and Van Hoogenhuyze and Verploegh[7] have reported for individuals on normal diets. The fluctuations are large. In Experiment 5, for instance, there is an increase from 53 to 81 mg. between two successive hours, an increase of over 50 per cent. Special care was used in such instances to check up the analytical work, determinations being made in triplicate wherever marked variations were found between two successive hours. The marked creatinine fluctuations recorded in Experiments 5, 6, and 7 are obviously not due to incomplete emptying of the bladder. In Experiment 8 (Table III), where 47.1 mg. of creatinine are eliminated for the period from 6 to 7 followed by 60.4 mg. from 7 to 8, it might be held that the bladder was not completely emptied at 7 o'clock. Reference to the total nitrogen will show, however, that this constituent practically doubled for the hour 7 to 8 as compared with 6 to 7, and as there is no relation between this increase and that of the creatinine and uric acid on a percentage basis, it is obvious that an explanation based upon incomplete emptying of the bladder is not tenable.

We believe that the experiments which are here reported demonstrate beyond doubt that for short fasts the creatinine output may show marked variations from hour to hour. In nearly all of our experiments there is a tendency for a very low creatinine elimination during 1 hour of the late afternoon or early evening. This point is also shown in the experiments recorded in Table III, in which the water intake is markedly different from the earlier ones. The uric acid output in these latter experiments is hardly as regular or constant as in Experiments 4 to 7. The effect of varying the water intake is not, however, clear-cut.

In conclusion, I wish to express my thanks to Dr. S. R. Benedict for his helpful suggestions throughout this work.

[6] Shaffer, P., *Am. J. Physiol.*, 1908–09, xxiii, 1.

[7] Van Hoogenhuyze, C. J. C., and Verploegh, H., *Z. physiol. Chem.*, 1905, xlvi, 415.

TABLE I.

Experiment 1. August 8. Fasting. Water Taken Every Hour. Quantity Not Measured.

Hour.	Volume.	Uric acid.
	cc.	*mg.*
7– 8	44	25.0
8– 9	25	18.7
9–10	27	21.5
10–11	19	14.4
11–12	14	11.7
12– 1	17	13.9
1– 2	18	11.5
2– 3	15	11.6
3– 4	12	9.7
4– 5	16	9.5
5– 6	12	9.6
6– 7	14	9.5
7– 8	22	7.1
8– 9	14	4.7
Total	269	178.4

Experiment 2. October 3. Fasting. Water Taken Every Hour. Quantity Not Measured.

Hour.	Volume.	Uric acid.
	cc.	*mg.*
7– 8	39	21.4
8– 9	50	11.5
9–10	98	23.0
10–11	69	18.7
11–12	71	15.6
12– 1	14	6.8
1– 2	41	10.0
2– 3	52	11.8
3– 4	12	5.8
4– 5	22	6.8
5– 6 } 6– 7 }	45	15.0
7– 8	70	9.9
8– 9	176	12.6
Total	759	168.9

Experiment 3. October 7. Fasting. No Water.

Hour.	Volume.	Hourly volume (average).	Uric acid.	Hourly uric acid (calculated).
	cc.	*cc.*	*mg.*	*mg.*
7–10	195	65.0	67.4	22.4
10– 1	61	20.3	42.8	14.2
1– 3.45	39	14.0	23.8	8.6
3.45– 6	36	16.0	27.2	12.0
Total	331		161.2	

TABLE II.

Experiment 4. October 24. Fasting. Water, 100 Cc. Each Hour.

Hour.	Volume.	Uric acid.	Total nitrogen.
	cc.	*mg.*	*gm.*
7– 8	64	20.6	
8– 9	93	26.8	
9–10	118	28.8	
10–11	122	18.7	
11–12	92	16.2	
12– 1	60	14.0	
1– 2	58	13.9	
2– 3	27	13.0	
3– 4	26	10.6	
4– 5	24	11.7	0.313
5– 6	14	7.1	0.185
6– 7	30	10.7	0.428
7– 8	23	9.9	0.324
8– 9	66	9.9	0.451
Total.....	817	211.9	

Experiment 5. November 2. Fasting. Water, 100 Cc. Each Hour.

Hour.	Volume.	Uric acid.	Creatinine.	Total nitrogen.
	cc.	*mg.*	*mg.*	*gm.*
7– 8	56	25.2	60.6	0.362
8– 9	148	24.0	60.7	0.441
9–10	62	21.8	60.0	0.415
10–11	38	19.0	49.8	0.300
11–12	37	15.4	49.8	0.302
12– 1	25	14.2	51.0	0.272
1– 2	36	15.4	49.8	0.364
2– 3	47	16.0	50.6	0.382
3– 4	54	19.0	64.0	0.418
4– 5	53	16.0	53.4	0.331
5– 6	58	23.4	81.0	0.517
6– 7	97	16.6	64.8	0.482
7– 8	49	16.8	53.4	0.343
8– 9	57	16.6	53.4	0.361
Total.....	817	259.4	802.3	5.290

TABLE II—*Concluded.*

Experiment 6. November 9. Fasting. Water, 100 Cc. Each Hour.

Hour.	Volume.	Uric acid.	Creatinine.	Total nitrogen.
	cc.	*mg.*	*mg.*	*gm.*
7– 8	42	25.3	52.0	0.394
8– 9	64	25.4	54.5	0.471
9–10	73	25.2	56.0	0.517
10–11	50	22.5	50.5	0.405
11–12	95	18.2	54.0	0.466
12– 1	53	17.2	52.8	0.393
1– 2	43	16.8	52.0	0.395
2– 3	35	16.4	50.5	0.376
3– 4	26	16.4	51.2	0.332
4– 5	35	17.0	53.1	0.404
5– 6	37	13.6	50.5	0.389
6– 7	25	10.5	37.6	0.276
7– 8	39	13.1	53.8	0.412
8– 9	68	17.5	59.1	0.556
Total.....	685	255.1	727.6	5.786

Experiment 7. November 16. Fasting. Water, 100 Cc. Each Hour.

Hour.	Volume.	Uric acid.	Creatinine.	Total nitrogen.
	cc.	*mg.*	*mg.*	*gm.*
7– 8	41	20.8	40.5	0.362
8– 9	41	20.8	50.5	0.440
9–10	50	27.8	54.0	0.504
10–11	32	16.8	41.6	0.377
11–12	62	17.0	48.0	0.482
12– 1	56	16.0	49.2	0.468
1– 2	34	14.8	48.4	0.376
2– 3	20	13.2	48.0	0.340
3– 4	23	12.7	48.2	0.383
4– 5	33	11.2	48.4	0.402
5– 6	41	10.6	51.2	0.476
6– 7	154	11.4	58.9	0.560
7– 8	31	10.8	42.6	0.315
8– 9	38	12.5	46.0	0.452
Total.....	656	216.4	675.5	5.937

TABLE III.

Experiment 8. December 5. Fasting.

Water: 100 cc. hourly to 2 p.m.
200 " at 2 p.m.
150 " hourly to 7 p.m.
250 " at 7 p.m.
200 " " 8 "

Hour.	Volume.	Uric acid.	Creatinine.	Total nitrogen.
	cc.	*mg.*	*mg.*	*gm.*
7– 8	67	24.1	57.3	0.420
8– 9	77	25.6	62.3	0.454
9–10	171	22.5	61.2	0.539
10–11	132	17.6	56.7	0.439
11–12	34	15.4	49.8	0.293
12– 1	37	16.0	56.8	0.358
1– 2	41	16.0	60.5	0.397
2– 3	66	13.5	54.0	0.375
3– 4	69	12.0	57.8	0.451
4– 5	93	9.0	52.0	0.426
5– 6	25	Lost.		
6– 7	26	8.6	47.1	0.256
7– 8	164	11.1	60.4	0.478
8– 9	275	9.8	57.8	0.533

Experiment 9. January 4. Fasting. Water, 50 Cc. Each Hour.

Hour.	Volume.	Uric acid.	Creatinine.	Total nitrogen.
	cc.	*mg.*	*mg.*	*gm.*
7– 8	73	25.0	60.6	0.505
8– 9	83	25.5	62.6	0.516
9–10	77	23.0	62.9	0.484
10–11	43	21.4	59.6	0.351
11–12	30	22.0	58.9	0.347
12– 1	22	18.8	59.2	0.312
1– 2	27	17.9	60.8	0.340
2– 3	16	12.5	50.0	0.230
3– 4	17	12.5	60.3	0.265
4– 5	14	9.6	55.5	0.237
5– 6	13	9.1	46.0	0.215
6– 7	22	17.3	65.6	0.332
7– 8	23	11.8	51.3	0.311
8– 9	19	9.8	41.6	0.273
Total.....	479	236.2	794.9	4.718

CORN AS A SOURCE OF PROTEIN AND ASH FOR GROWING ANIMALS.

By ALBERT G. HOGAN.

(From the Department of Chemistry, Kansas State Agricultural Experiment Station, Manhattan.)

(Received for publication, February 10, 1917.)

Agriculturists have long since recognized the inadequacy of the corn kernel as the sole diet of growing animals, and investigators in the field of nutrition have made numerous studies to determine more exactly the nature of its deficiencies. An earlier publication of the author presented some recent data showing that the first limiting factor in corn for the growth of young rats is the lack of certain inorganic constituents. When the mineral deficiencies were corrected, however, normal growth could not be secured, even after the addition of considerable quantities of purified protein, indicating a lack of suitable growth accessories. McCollum and his collaborators have presented data bearing on this problem, and state that the maize kernel is lacking in one of the accessories, which they have designated as fat-soluble A.

Our earlier investigations disclosed some minor variations in the way swine and rats responded to an exclusive diet of maize. For example, the mineral deficiencies of corn were tolerated much better by the swine, while the protein deficiencies were tolerated much more easily by the rats. Assuming that corn is poor in one or more of the growth accessories, swine are affected much less injuriously by this than are the rats.

Our earlier work has been extended, and efforts are now being made to determine specifically what inorganic elements in the ash and what amino-acids in the proteins are deficient in quantity, and thus constitute limiting factors when the corn kernel is used as the exclusive diet of young animals. Data from this laboratory have been presented[1] showing that the addition of trypto-

[1] Hogan, A. G., *Proc. Am. Soc. Animal Production*, 1916 (in press).

phane and lysine improved the proteins of corn, but our results at that time did not indicate which of these amino-acids was the first limiting factor in the protein mixture of the corn kernel. Our later results have established that point.

The methods used in our earlier work were followed closely in these feeding trials, and in most instances rats were used as experimental animals. In some cases swine were used, but only to a limited extent.

I. The Inorganic Limitations of the Corn Kernel.

Because of the inadequacy of the proteins in corn they were supplemented by a comparatively ash-free protein, a specially prepared specimen of egg white. The analyses of corn make it probable that the animals receiving this grain as an exclusive ration will first require calcium to permit the normal nutritive processes, and this view seems upheld by the facts. One lot of animals received corn plus the ash-free protein, and other lots received this same ration with various mineral supplements. In one case the supplement was a complete ash mixture;[2] in another it was calcium lactate, and in another potassium phosphate. In the two latter cases the animals received the same quantities of calcium lactate and potassium phosphate as the first lot received in the complete ash mixture. During the period of observation the lot receiving the complete ash mixture grew somewhat more rapidly than that receiving corn plus the calcium lactate. The rats receiving corn plus potassium phosphate profited in no way by the addition, and died about as quickly on the ration as those receiving no mineral supplement whatever. The results appear in Table I. Rats were used as experimental animals.

	gm.
[2] Ca lactate	468.0
K_2HPO_4	280.8
NaCl	123.1
Na citrate	31.2
Fe citrate	23.8
$MgSO_4$	31.7

TABLE I.

Mineral Supplements for Corn.

Ration.....	Corn + ash-free egg white.		Corn + ash-free egg white + K_2HPO_4.		Corn + ash-free egg white + Ca lactate.		Corn + ash-free egg white + complete ash mixture.	
Sex and No....	♀ 1	♂ 1	♀ 3	♂ 3	♀ 2	♂ 2	♀ 2	♂ 1
Time.	Average weight, *gm.*							
wks.								
Initial.	50	52	46	49	50	49	66	56
3	64	78	50	48	71	82	95	85
6	50	72	60	57	75	98	102	107
9	Dead.	Dead.	Dead.	57	85	116	114	132
12				Dead.	89	124	124	177
15						132	125	180
18						146	130	175
21							135	215
24							135	225
27								225

II. The Amino-Acid Deficiencies of Corn Proteins.

Of the individual proteins of corn, zein has received the most attention from investigators. According to Osborne and Mendel (1914, *a*), it makes up about 40 per cent of the total nitrogenous constituents. It contains neither lysine nor tryptophane, and as has been known for some time, is unable to support life when it forms the only protein of the ration. Willcock and Hopkins used young mice in feeding trials, and obtained some of the earlier data bearing on this point. When receiving a diet containing no other protein than zein, their mice lived only a short time. When tryptophane was added to the ration, life was much prolonged. Some years later Osborne and Mendel (1914, *b*) showed that zein could support life and even permit growth if it were supplemented with lysine and tryptophane,

Although there is no question that these amino-acids must be added to zein in order that it may support growth in the animal organism, it is not so certain that the mixture of proteins in the entire corn kernel would show a corresponding deficiency. It has been demonstrated by Osborne and Mendel (1914, *a*) that corn glutelin is an adequate protein, and there is a general im-

pression that the proteins of the corn germ are relatively very effective. Some data in support of that view have been obtained in previous years at this Institution. One lot of swine received corn alone. Other lots received corn with the addition of various supplements, including casein and corn germ. Table II gives further details concerning the rations, as well as the growth of the animals.

TABLE II.

Corn Germ as a Source of Protein for Growing Swine.

Lot No.	36	29	34	23	31
Nutritive ratio	1:6	1:6.9	1:8.8	1:8.0	1:8.8
Ration	Corn + casein + salt mixture.	Corn + casein.	Corn + starch + casein + salt mixture.	Corn + corn germ.	Corn + salt mixture.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Protein { corn	10.29	11.39	5.64	7.74	10.88
Protein { supplement	4.21	1.22	4.78	5.08	
Ether extract	4.97	4.19	3.02	7.20	4.58
Nitrogen-free extract	76.15	81.40	82.80	77.20	80.30
Ash	4.33	1.71	3.73	2.79	4.27
	lbs.	*lbs.*	*lbs.*	*lbs.*	*lbs.*
Average initial weight	21	31	24	31	25.3
Average final weight, (180 days)	200	142	165	150	37.6

Lots 29 and 23 received tap water, high in calcium. The others drank only distilled water. There were three animals in each lot.

The table presents in a striking way the vast superiority, for growth production, of casein over the corn proteins. For all practical purposes it may be said that Lots 29 and 31 received the same diet, with the exception that casein to the extent of only 1.22 per cent of the entire ration was added to the feed of Lot 29. Yet this comparatively small addition changed the ration from one that permitted practically no growth to one that permitted approximately two-thirds normal growth. Possibly a suitable mineral supplement would have augmented this difference, for obviously Lot 29 was receiving no excess of mineral matter. A comparison of Lots 31 and 34 shows the superiority

of the casein in an equally convincing manner. Each lot received the same amount of digestible protein, assuming that the casein was entirely digestible. The fact that half this protein was casein, however, changed the ration from one that would barely sustain life to one that permitted a degree of growth not far from normal. Brief consideration of Lots 23 and 34 is also of interest. A strict comparison is impossible as the composition of the rations is quite dissimilar. Obviously, however, the proteins in the corn germ are effective in supporting growth of young animals. It would not be impossible, therefore, that the proteins of corn germ contained high percentages of the amino-acids lacking in zein, and might at least in some measure compensate for the deficiencies of the latter protein.

In our earlier work lysine and tryptophane were added to finely ground corn, and this mixture was fed. This addition had no apparent value, but the results were not considered decisive because rats failed to grow notably better when our purified proteins, such as egg white, were added to corn. It seemed evident therefore that some other factor than the quality of protein should be considered. This may have been a suitable mineral supplement, growth accessories, or some less considered question. In order to obviate all such possibilities, it seemed desirable to use a ration known to be adequate in every respect other than protein. For that purpose a ration[3] approximating some published by Osborne and Mendel was adopted. At first the rats received approximately 9 per cent protein, but later this was increased to 12 per cent. Corn itself seldom contains much over 10 per cent protein, so in order to have 12 per cent in the ration after admixture with other constituents, it was necessary to obtain the protein in a more concentrated form. Corn gluten did not seem suitable, as it lacks the corn germ. Accordingly efforts were made to obtain a product in which the natural mixture of

[3] The ration was as follows.

	Protein.	
	9 per cent.	12 per cent.
	gm.	*gm.*
Corn protein	285.0	360.0
Protein-free milk	250.0	250.0
Butter	250.0	250.0
Starch	260.0	138.0

corn proteins was modified as little as possible, but which contained them in larger proportion. The corn was ground to an impalpable powder, and boiled in a large volume of water made slightly acid with acetic acid. The purpose was to coagulate the soluble proteins, and at the same time bring the starch into a colloidal solution. After standing some time the protein settled and then the supernatant liquid was siphoned off. This process was repeated five or six times, until the liquid did not give the iodine test for starch. The product obtained in this way contains 30 to 35 per cent protein, and is suitable for use in the feeding mixture mentioned above.

At the beginning of the feeding period, one lot of rats received the mixture containing the prepared corn proteins, and another lot of six received the same ration with the addition of lysine and tryptophane. The amount of corn protein in this basal ration was purposely made very low, in order that growth should occur very slowly. Under these circumstances, any increase in the rate of growth due to the added amino-acids should be clearly perceptible. The amounts of these supplements were relatively small; to each 1,000 gm. of the feed, 3 gm. of tryptophane and 3 gm. of lysine dichloride were added. Two of the rats receiving the mixture containing no added amino-acids died relatively soon, and another died near the close of the experiment. These are not included in the charts. None of the rats receiving the additional amino-acids died, and though they did not grow rapidly, yet in the first 16 weeks they made over three and a half times the gain that the other lot did. At the end of 17 weeks, the lysine and tryptophane were removed from the diet, and these rats promptly began losing weight. After a short interval, tryptophane was added to the diet of three of these animals, and both lysine and tryptophane to the ration of the other three. All began growing again, but those receiving lysine and tryptophane grew much more rapidly than those receiving only tryptophane. Later the animals were given the basal ration with lysine alone added, and without exception they lost weight at once, showing clearly that tryptophane is the first limiting factor in the corn proteins. After these proteins are supplemented with tryptophane, the addition of lysine again accelerates the rate of growth, and the latter amino-acid is evidently the second limiting factor. More complete data are presented in Charts 1, 2, and 3.

EXPLANATION OF CHARTS.

All animals received the same basal ration at all times. At the point indicated by *a*, the protein was increased from 9 per cent of the ration to 12 per cent; the point *b* indicates a further increase to 15 per cent protein. In addition to the basal ration, two of the animals shown in each chart received at various times lysine and tryptophane, given together and separately. The numbers in the upper left hand corner signify which animals received the amino-acids, and the heavy lines accompanying them indicate the time during which these supplements were administered. A gap in a line shows that none of that particular amino-acid was received during the interval thus indicated. It is evident from inspection of these charts that the addition of lysine alone did not cause the rats to grow more rapidly. The animals did grow more rapidly, however, when tryptophane alone was added, and still more rapidly when both amino-acids were added.

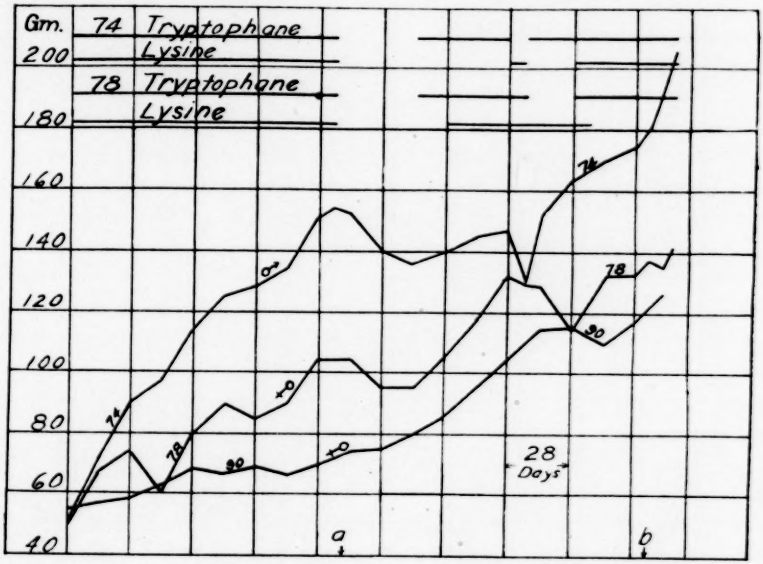


CHART 1.

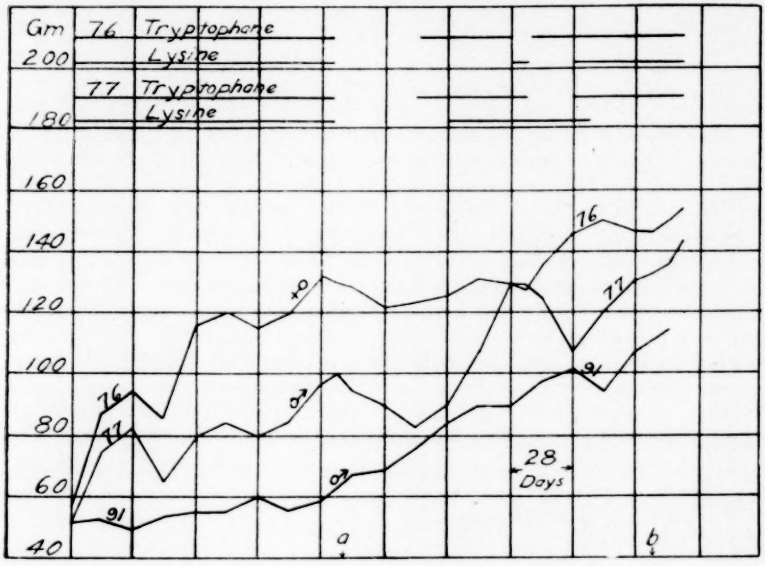


CHART 2.

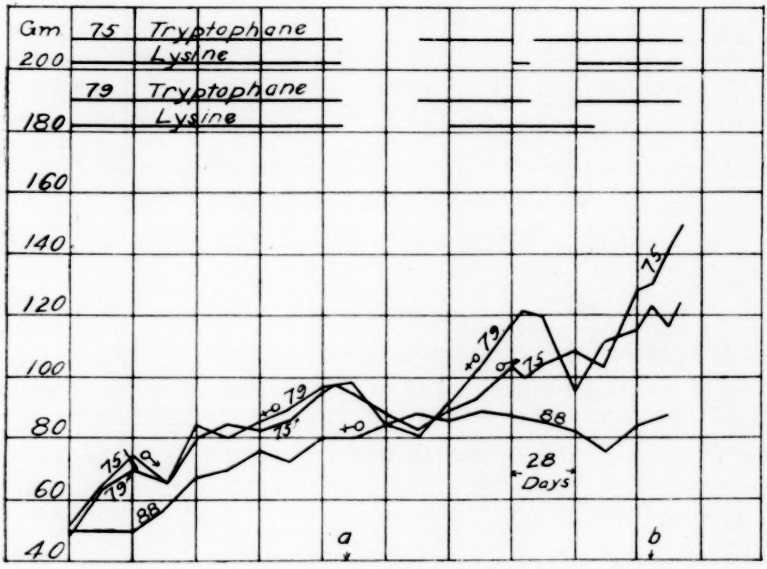


CHART 3.

SUMMARY.

The most important mineral deficiency of corn is calcium. Tryptophane is the first limiting factor in the proteins of the corn kernel, and then lysine.

BIBLIOGRAPHY.

Hogan, A. G. (1916), *J. Biol. Chem.*, xxvii, 193.
McCollum, E. V., Simmonds, N., and Pitz, W. (1916–17), *J. Biol. Chem.*, xxviii, 153.
Osborne, T. B., and Mendel, L. B. (1914, *a*), *J. Biol. Chem.*, xviii, 1.
Osborne and Mendel (1914, *b*), *J. Biol. Chem.*, xvii, 325.
Willcock, E. G., and Hopkins, F. G. (1906–07), *J. Physiol.*, xxxv, 88.

THE CHEMICAL NATURE OF THE "VITAMINES."

III. THE STRUCTURE OF THE CURATIVE MODIFICATIONS OF THE HYDROXYPYRIDINES.

By ROBERT R. WILLIAMS.

(From the United States Department of Agriculture, Bureau of Chemistry, Washington.)

PLATE 4.

(Received for publication, February 28, 1917.)

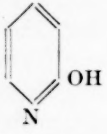
In the first paper of this series (1) it was shown that α-hydroxypyridine exists in two crystalline forms differing in physiological properties. These were assumed to be distinct isomeric substances. Moreover, evidence was found that the sodium salt of the substance represents a third (enol) form. One of the modifications was obtained as needles and possessed remarkable curative power for polyneuritis in pigeons, but under ordinary conditions spontaneously changed into crystalline granules which were physiologically inactive. Such a change of physiological properties appears to take place under similar conditions in all the mono- and polyhydroxypyridines so far studied.

In order to be of value in elucidating the chemical nature of the "vitamines" occurring in foodstuffs, the discovery of this physiologically important transformation must be supplemented by definite evidence of the chemical structure of the curative form. It will further be necessary to distinguish sharply between those structural features which are indispensable to the antineuritic property and those which play a secondary, insignificant, or adverse rôle. Some initial steps in this direction constitute the main subject of the present paper.

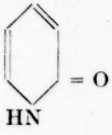
Structure of the Curative Substance.—The difference in physiological activity in the two crystal forms is sufficient to prove that they are distinct substances. Analyses and molecular weight determinations confirm the earlier assumption that they are iso-

meric. All the evidence indicates that the stability of each form is determined by temperature, solvent, etc., and that the two modifications are readily interconvertible by proper adjustment of conditions. These facts suggested a labile hydrogen atom.

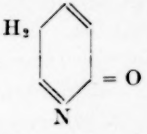
That there should exist some form of lability of the hydrogen atom of the hydroxyl group of α-hydroxypyridine seems wholly probable if one may reason by analogy to phenol. In fact a lability of this atom appears to have been proven by the production of two isomeric alkyl derivatives, in one of which the alkyl radical is attached to oxygen and in the other to nitrogen (2). On the other hand, there is no reason to suppose that any other atom in the molecule shows a similar lability. Therefore it is assumed *that all the isomeric rearrangements of α-hydroxypyridine involve the shifting of only one atom; i.e., the hydrogen of the putative hydroxyl group.* If the truth of this assumption is granted, the compound theoretically may exist in six isomeric forms as follows:



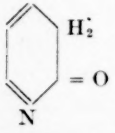
I. Enol.



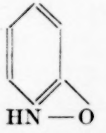
II. Pyridone.



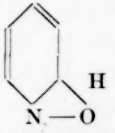
III. Para-quinonoid.



IV. Ortho-quinonoid.



V. Pseudo betaine.



VI. Anthranil.

Two of these formulas represent the hydrogen as attached to nitrogen, three as attached to carbon, and one as attached to oxygen.

By a study of the methyl ethers of α-hydroxypyridine it was possible tentatively to eliminate Formulas I, III, IV, and VI as representative of the curative form, for it was found that while methoxypyridine is non-curative and toxic, the nitrogen methyl derivative shows distinct indications of curative properties.

It has been definitely proven that in the so called methylpyridone, the methyl group is actually attached to nitrogen

since methylamine is produced by reduction with sodium amalgam (2). But formulas corresponding to both II and V have been assigned to this compound (3) and the adherents to the respective theories have not recorded any agreement on the question. Kauffmann regarded the structure shown in Formula V as definitely proven by the fact that the vapors of the substance, under the influence of the Tesla current, glow with a blue luminescence similar to that produced by pyridine and benzene under the same conditions (4). He looked upon this as conclusive evidence of the presence of three pairs of shifting double bonds. Actually this evidence does not exclude the possibility of the existence of two forms in equilibrium. We have equally definite evidence that the substance does exist in a second form in the fact that it is strongly basic and forms well defined salts with acids. Such properties are impossible in a compound in which the only nitrogen atom is already pentavalent. Further reference will be made to this matter later.

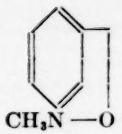
In view of the existing uncertainty regarding the structure of methylpyridone it was impossible to decide between Formulas II and V for the curative form of α-hydroxypyridine, even if the tentative exclusion of the remaining formulas should prove valid. Formula II has been generally accepted for the needle (curative) form, which is the only crystallographic modification heretofore observed. But the literature (5) does not describe a single experimental fact which cannot be interpreted according to either formula, and no additional chemical or physical evidence bearing on the subject has been found as yet. However, the physiological properties of β-hydroxypyridine (6) render a conclusion possible regarding the structure of the curative form of the free and substituted hydroxypyridines, regardless of the existence or structure of other forms. A structure corresponding to Formula II is obviously impossible for β-hydroxypyridine, as has been pointed out by Pechmann and Baltzer (2), since the valences of all the atoms of the ring cannot be satisfied on this assumption. The fact that β-hydroxypyridine under proper conditions shows physical, chemical, and curative properties similar to those of the α derivative therefore enables us to exclude Formula II and come to a decision that the curative forms of all the hydroxypyridines possess a structure best represented by formulas of type V, a

conclusion which is confirmed by further evidence presented later in this paper.[1]

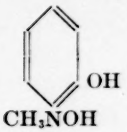
If β-hydroxypyridine exists in two forms and the structure of the curative modification is of type V it is safe to predict the existence of a second methyl derivative VII (7). A further study of the chemical and physiological properties of β-hydroxypyridine is in progress and all experimental data regarding it will be reserved for later publication.

Structure of Non-Curative Modifications.—Pending further work the structure of the non-curative forms of the hydroxypyridines and the alkyl pyridones remains in doubt. We may reasonably conclude that the crystalline non-curative forms of monohydroxypyridines are not enols since they do not instantaneously absorb bromine in cold alcoholic solution. Whether they are derivatives of the pyridone (II) or of the para- (III) or of the ortho- (IV) quinonoid type it is impossible to say, though the predominance of para-quinonoids in the benzene series would argue in favor of Formula III. Formula VI seems altogether improbable. In view of the experimental evidence described in the literature, supplemented by physiological tests referred to in the experimental part of this paper, it seems probable that methylpyridone exists in a betaine-like form (V) and also in the form of a hydrate (VIII) or possibly an isomer (IX) or both. The difficulty with which the substance is freed from water suggests the hydrate (8).

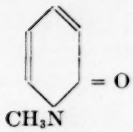
[1] It will be understood that the possible imperfections of the conventional methods of representing chemical structure are not taken into account in this conclusion. There is much to suggest that the dimorphism of α-hydroxypyridine may be akin to what has been loosely called "physical isomerism." In the writer's opinion this conception is in itself a contradiction in terms. If two solid modifications of any substance such as tin or phosphorus possess different proportions of free or available energy, they must differ from one another structurally. In the absence of evidence to the contrary the free chemical energy must be presumed to arise from the same source as do those forces which we speak of as valences. That is to say, the free energy must be in the molecule, not deposited upon it.



VII



VIII



IX

Betaine-Like Character of the Curative Form.—If the structure of the curative form of α-hydroxypyridine is indicated in Formula V it is essentially a betaine. Experimental results indicate that there is an analogy between the two crystalline forms of α-hydroxypyridine on the one hand and the α-amino-acid esters and their corresponding betaines on the other in respect to their mutual convertibility at various temperatures. Willstätter (9) has found in the case of the methyl ester of dimethylamino-acetic acid and the isomeric betaine that both forms are stable below 135°; between 135° and 293° the betaine form exists to the exclusion of the ester, and above 293° the ester becomes more stable than the betaine. A similar relation according to Willstätter exists in all the α-betaines. In α-hydroxypyridine the interconversion of the two forms apparently takes place with greater ease than the rearrangements studied by Willstätter but are probably of a similar nature, as the former involves the shift of a relatively labile hydrogen atom as compared with the less mobile alkyl groups with which Willstätter was concerned. The conversion of methylpyridones into corresponding methoxypyridines by heat (10) also presents a close analogy to α-betaines in which the methyl group may similarly migrate from nitrogen to oxygen. The hypothetical formation of a hydrate of methylpyridone in water solution also conforms to the chemistry of betaines.

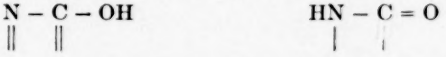
In view of this similarity it seemed wise to attempt a physiological study of some compounds, the betaine structure of which is generally accepted. Some years ago the author made a few tests of the antineuritic properties of trigonelline (hydrate) which resulted entirely negatively (11). Drummond and Funk (12) also reported that betaine (trimethyl glycine) salts are devoid of curative action. But on closer inspection it will be seen that these experiments are not conclusive evidence on the point in question, since in the classes of betaines as a whole the characteristic be-

taine ring is not known to persist in water solution nor in the hydrated crystalline forms, and certainly does not exist in the salts of these bases (13). Betaines are essentially anhydrides which take up water (or acids) with greater or less ease. In view of this fact it seemed unlikely that any antineuritic properties which the commoner betaines might possess in the anhydride form could actually be demonstrated, since physiological processes take place in aqueous media. Notwithstanding this obvious difficulty a few birds were treated by the expeditious injection of freshly made cold water solutions of dehydrated trigonelline and betaine. The results obtained with trigonelline under these conditions were inconclusive. With betaine it was found possible to effect substantial improvement or practically complete cures of the paralytic symptoms in nearly all cases, so that no doubt can exist that the substance is curative to a degree at least as long as the anhydride form persists.

The Feature Essential to Antineuritic Properties.—The curative action of betaine is important, first, in confirming the structure of the curative form of the hydroxypyridines and, second, in showing that a labile hydrogen atom is not essential to such properties. That a subordinate or negligible rôle is played by the labile hydrogen atom is further indicated by the results obtained with methylpyridone and trigonelline. The distinct beneficial effects upon polyneuritis of betaine as well as of allantoin and certain purines and pyrimidines (14), and the evidence cited in the second paper of this series bearing on the antineuritic properties of adenine (15), indicate that the pyridine ring is not requisite to some such physiological properties. Judging from curative tests alone one might infer that a betaine-like structure is an essential and highly important feature of natural "vitamines." However, attempts to protect birds against polyneuritis by the regular administration of methylpyridone, trigonelline, or betaine have failed. This suggests that the response of polyneuritic birds to treatment with such substances is a phenomenon not intimately connected with true antineuritic action. But such a view seems less reasonable than that the failure of the synthetic substances in protective experiments is due to the extreme instability of their active forms, their toxicity, or other properties which render them incompletely adaptable to the uses of the

animal organism. Accordingly, it is assumed as a working hypothesis that such a betaine-like structure constitutes an essential part of "vitamine" molecules.

Distribution of Betaine-Like Ring in Nature.—If this reasoning proves to be correct, it will still fail to assist greatly in establishing the identity of the natural "vitamines," for a betaine-like structure is theoretically possible in a large proportion of the known nitrogenous constituents of animal tissue. It may exist in any substance containing a basic nitrogen atom in the proximity of a labile hydrogen atom of an alcohol, phenol, carboxyl, or amino group. Aside from the amino-acids and their alkyl substitution products, for many of which both betaine and straight chain modifications have been proven, there is another much larger class of compounds in which a betaine structure is possible. It includes all the amino and oxy derivatives of cyclic nitrogen compounds, of which the oxypyrimidines may be regarded as representative. In some of these substances a shifting of hydrogen from oxygen to nitrogen has been proven by the formation of both oxygen and nitrogen ethers. This form of tautomerism commonly has been expressed as follows:



However, all the facts would be accounted for equally well by the assumption of the existence of the following modifications:

In most instances no purely chemical means would be adequate to determine which conception is correct and neither can be dismissed as improbable. Modifications of either or both types may exist. The work of Hans Meyer (16), of Geake and Nierenstein (17), and of Griess (18), and the polymorphism of pseudo conhydrine (19) and 4-phenyl-isocytosine (20) are of interest in this connection. To determine definitely in what compounds and under what conditions betaine modifications actually exist may require many years of work. To decide which of such

compounds are sufficiently stable to be absorbed unchanged from the alimentary tract may prove another equally difficult problem. Progress in both cases would be greatly facilitated by the discovery of some physical means of detecting the betaine linking.

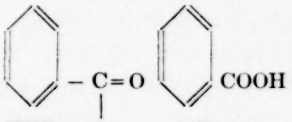
Nicotinic Acid.—While it is probable that most potential betaines do not function as "vitamines" in our foodstuffs, the assumption that the "vitamines" invariably belong to a narrowly restricted group of compounds is without adequate foundation. Evidence has been found suggesting that an isomeric form of adenine is responsible for antineuritic properties in a certain fraction of autolyzed yeast (15). Should this prove to be correct the writer still would not presume that such a form of adenine is necessarily the only antineuritic substance in yeast, much less in all nature.

Vedder and Williams (21) fractioned the filtrate from the decomposed phosphotungstates of hydrolyzed extract of rice polishings by precipitating with silver nitrate, first in neutral solution (purine fraction) and second in alkaline solution (Funk's "vitamine" fraction). The filtrate from the second precipitate constituted a third fraction. All these fractions protected fowls from polyneuritis, although only the second, or "vitamine" fraction, produced prompt cures of the disease. They recorded their conclusions as follows: "Therefore it appears certain that there are several groups of chemical substances that are capable of protecting fowls against polyneuritis gallinarum."

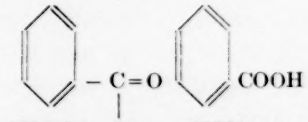
This is of particular interest in view of the obvious discrepancy in elementary composition between adenine and the substances obtained by Funk from his "vitamine" fraction of yeast and rice polishings. Adenine contains more than 50 per cent of nitrogen while the relatively pure crystalline substances from Funk's fraction, among which was nicotinic acid, contained only 10 to 12 per cent of this element. The possibility that the curative substance in Funk's "vitamine" fraction was actually a derivative of a tautomeric form of adenine seems very remote. In view of these considerations one can hardly escape the feeling that the presence of nicotinic acid in considerable proportions in Funk's "vitamine" fraction of both yeast and rice polishings is not accidental, and that Funk's conjecture, "It is not unlikely

that nicotinic acid is a decomposition product of the vitamine" (22) had some foundation in fact.

Turnau (23) has described a series of basic salts of the α- and γ-pyridine carboxylic acids and their methyl betaines. Thus a water solution of picolinic acid, to which is added an excess of hydriodic acid, on evaporation deposits a well defined basic hydriodide to which Turnau assigned the formula X. That is, he regards it as the hydriodide of picolinic acid picolinate. The corresponding methyl derivative XI possesses an obvious resemblance to a betaine.

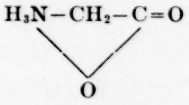


X

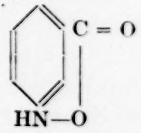


XI

Turnau did not succeed in preparing such derivatives of nicotinic acid but his work suggested the possibility of the existence of an isomer, polymer, or simple derivative of nicotinic acid in which the carboxyl group exists as a betaine ring. Whether or not Turnau's formulas are correct, if the aliphatic α-amino-acids under any conditions have the structure XII, there is no theoretical obstacle to the existence of nicotinic acid in the form represented by XIII, either free or in some simple combination, especially since both the methyl betaine and methyl ester of nicotinic acid are known.



XII



XIII

Accordingly, endeavors were made to convert nicotinic acid into an antineuritic form. On decomposing nicotinic acid hydriodide with silver oxide (24), the filtrate from the silver halide was found to possess marked curative properties. These results are such as to encourage further investigation with a view to the

preparation and isolation of such betaine derivatives, and finally to proving or disproving the "vitamine" character of nicotinic acid as it exists in certain antineuritic foodstuffs.

EXPERIMENTAL PART.

Pigeons were used as experimental animals, polyneuritis being developed by feeding exclusively on white rice. For the curative experiments described below birds were selected which had developed characteristic symptoms of the disease and showed these symptoms continuously in a severe form for several hours before treatment. This was done since it has been found that polyneuritic fowls sometimes suffer from mild, brief, or intermittent attacks of paralysis and subsequently recover without treatment, eventually developing chronic symptoms accompanied by emaciation and general weakness. Birds exhibiting the chronic form of the disease whether preceded by brief acute attacks or developing progressively were also excluded, since such birds cannot be treated with uniform success by any measures. Under this plan, of the total number of pigeons fed on white rice, only about 50 per cent were available for use, the losses including those birds which developed acute symptoms and died suddenly and those which failed to show severe, persistent paralysis within a reasonable time, usually about 35 days. The birds varied greatly in the incidence of the more characteristic type of the disease, a fact which has been insufficiently emphasized in recent literature. The writer has been unable to form a definite opinion as to the correlation of this incidence with the age, sex, appetite, or habits of pigeons, or with the precise degree of decortication of the rice used for feeding, or the season of the year chosen for experimentation. Any advantage which may be gained by forced feeding appears to be more than offset by the complications resulting from stuffed crops and excessive rough handling.

α-Hydroxypyridine.—Some further observations (1) have been made upon the conditions under which the two crystalline forms of α-hydroxypyridine may be transformed into one another. If the needle form is desired, it has been found advisable to heat the pure 6-hydroxynicotinic acid used for its preparation in an

oven at a temperature of 140° for several hours. The acid is then distilled in a flask previously thoroughly dried by baking and collected in a dry receiver protected from the moisture of the atmosphere by a calcium chloride tube. Under these conditions the colorless oily distillate remains in a clear fluid state for some minutes, especially if its volume is large. Crystallization gradually takes place largely in the form of needles often 2 or 3 cm. in length. Some granular crystals have always been obtained even under these conditions, especially in the first few drops of distillate. That part of the mass crystallizing in granules is opaque while the portions in which needles predominate remain semi-transparent sometimes for days and detached fragments have a sticky character. During the course of a few days the amount of granular crystals grows from the points of original contamination as evidenced by the spread of opacity and the decrease in the stickiness of detached crystals.

Masses of either modification if redistilled (boiling point 280–282°) do not undergo change of crystal form to any great extent. This would seem to indicate relative stability of both forms at that temperature. On the other hand, if the granular form is fused and the superfused mass is kept at 70–90° till crystallization is complete, the needle form is obtained exclusively. But if by sudden cooling to lower temperatures the deposition of the granular form once begins in such a superfused mass the whole mass will granulate rapidly and any needle crystals which have previously grown in the magma will be converted almost instantaneously into granular aggregates of needle shape, indistinguishable to the naked eye from true needle crystals. These facts indicate that at a temperature of about 70–90° the needle form is the more stable. On the other hand, at room temperature conditions are reversed and the granular form is stable, as will be seen below.

In solution the relative stability of the two forms has proven a baffling problem. From solutions of either form in ethyl or amyl alcohol, chloroform, or ether it has been found possible to obtain only the granular form. Crystallization always takes place slowly from such solvents so that presumably there is opportunity for rearrangement during the process.

If either form is dissolved in boiling benzene, and ligroin is

added to the cooled solution a milkiness is immediately apparent (25). If crystallization can be induced within a few moments needles will predominate, occasionally to the exclusion of the granular crystals. For some unknown reason the needle crystals can be more readily obtained in considerable proportions when the operation of recrystallization is carried out upon several grams of substance at once. If crystallization takes place slowly only the granular type is obtained. However, commonly, even with the greatest care, it is impossible to avoid the simultaneous formation of both types. When this occurs the needles first formed are rapidly converted into the granular condition if allowed to remain in the benzene-ligroin mixture and especially if stirred. Microscopic fields of the needle type slightly contaminated with granular crystals have frequently been seen to undergo complete transformation within a few minutes (Fig. 1). Dr. F. E. Wright of the Geophysical Laboratory kindly examined the two forms of crystals and watched the progress of this transformation. He summarized his observations as follows:

Stable Form.—This occurs either in irregularly shaped grains or in branching aggregates of short crystals with pyramidal terminations and so grouped that the crystals intersect at right angles. Under the microscope each crystal is seen to be twinned in such manner that the planes of optic axes of any two superimposed twinned portions intersect at right angles. No cleavage was observed. The crystals are colorless and strongly birefracting. The refractive indices are approximately: $\alpha = 1.61$, $\gamma = 1.78$. The optic axial angle 2E is large, at least 140°; optical character, negative; axial dispersion strong $2E_r > 2E_r$. On many of the sections the acute bisectrix and one optic axis appear in the field in convergent polarized light. So far as can be determined the interference figure shows a symmetrical distribution of the interference colors, as though the crystal system were monoclinic with inclined dispersion. Many of the crystals contain liquid inclusions with movable gas bubble.

Unstable Form.—This crystallizes in acicular lath-shaped crystals which, however, are not stable in the solution (*i.e.*, a benzene-ligroin mixture) but are rapidly dissolved and replaced by the stable form. The process of inversion appears to be largely one of actual solution with immediate precipitation, along the dissolving crystal, of grains of the stable form. The inversion does not appear to be a direct change in the crystal state from the unstable form to the stable form. The optical properties so far as determined are: optical character, negative; extinction parallel with positive elongation and with plane of optic axis parallel to elongation; birefringence medium to fairly strong, less than that in the stable phase.

If the crystals are quickly removed from the benzene-ligroin mother liquor and dried, the mixed forms persist for a longer time, but even in a solid state and in a dry atmosphere the conversion of needles into granular crystals continues to occur, though at a much reduced rate. The needles become merely roughly needle-shaped granular aggregates in the course of a few days or at most weeks. Intimate mixing of the two forms appears to hasten the change, which seems to proceed from points at which granular crystals exist. Accordingly, the rate of change varies over a wide range.

Water solutions of the needles remain physiologically active for at most only a few days when kept at ordinary temperatures. At boiling heat the curative property disappears promptly. When such solutions, whether old or freshly prepared, are evaporated, a residual syrup remains which solidifies to a crystalline mass. If crystallization takes place at ordinary temperature in a desiccator the crystalline substance is non-curative, but if maintained at 70–90° the syrup slowly deposits needles which are curative. The granular crystals may be dried at ordinary temperature over phosphorus pentoxide but do not thereby become curative. Both forms of crystals appear to melt at 106–107° and admixture of the two forms does not depress the melting point. Probably the two modifications do not actually possess the same melting point but no distinction can be made because the substances are interconvertible. The polymorphism of the compound has a striking resemblance to that of conhydrine and pseudo conhydrine (19), and transformations take place with such ease as to make impossible a chemical differentiation of the hypothetical isomeric modifications. Even the preparation and isolation of the metastable form require conditions beyond the complete control of the experimenter who must expect failures more frequently than successes. The tendency of the stable modification to form acicular aggregates is very deceptive. Observation under crossed Nicol prisms is useful for the detection of this phenomenon. Persistence of crystal form when moistened with a benzene-ligroin mixture may also be taken as satisfactory evidence of the presence of the stable phase.

An amount of the needle crystals was obtained in apparently a nearly pure condition by crystallization from benzene and li-

groin, as above described. The crystals were quickly collected on a suction filter, washed with petroleum ether, and dried for an hour *in vacuo* over sulfuric acid and paraffin. A weighed sample was used for the determination of the molecular weight by the depression of the freezing point of water.

0.5200 gm. substance in 9.6164 gm. of water depressed the freezing point 0.868°. Molecular weight found: 108.9. Calculated for C_5H_5NO: 95.05.

This determination is in substantial agreement with one made by Koenigs and Geigy (25) by the vapor density method. The crystal form of the substances as described by these authors was that of needles.

After completion of the cryoscopic determination a portion of the solution used for this purpose was administered to a polyneuritic pigeon by injection. A dose of 1 mg. of substance had completely relieved the paralysis by the following morning.

A sample of the granular form prepared by crystallization from benzene and ligroin as above described was washed with petroleum ether, pressed between filter papers, kept *in vacuo* over paraffin for 24 hours, and dried over phosphorus pentoxide for a week.

In a solution of 0.3787 gm. substance in 9.1535 gm. of water a depression of the freezing point of 0.744° was observed. Molecular weight found: 106.4. Calculated for C_5H_5NO: 95.05.

Doses of 2 mg. of these crystals were administered to each of two polyneuritic pigeons by intramuscular injection. No improvement ensued and both birds died within 24 hours.

Nitrogen determinations on both forms were made by the Kjeldahl-Gunning-Arnold method under conditions which have been found satisfactory for the determination of nitrogen in a variety of pyridine derivatives.[2] The results did not agree very satisfactorily, suggesting that the substances were not completely dried by the methods used. However, since more than 14 per cent of nitrogen was found in duplicate determinations upon

[2] Personal communication of Dr. E. K. Phelps and Mr. H. W. Daudt of the Bureau of Chemistry. These determinations were kindly made by Mr. Daudt.

each modification, it is apparent that neither is a hydrate. This is further shown by their interconvertibility in the absence of water.

It is impossible to predict accurately the onset of polyneuritis in birds or to keep them alive very long after the severe symptoms have appeared, and the preparation and preservation of pure and well defined crystal forms of α-hydroxypyridine are equally precarious undertakings; consequently the physiological tests of these substances presented many difficulties. However, the findings recorded in a previous paper (1) have been verified in every practicable way by curative tests, so that no doubt can remain that the antineuritic properties are associated only with the needles.

An attempt was made to conduct a protective experiment with the needle form on pigeons fed on white rice. The necessity of making repeated attempts to obtain small amounts of the pure needle form daily involved so much labor that the experiment was discontinued after 3 days. Four pigeons (Nos. 137 to 140, inclusive) were fed with white rice and given by mouth 5 mg. daily of the granular form. Within 3 days the birds were seen to be slightly stupid as compared with controls. On the 8th day the feathers of all the birds were ruffled and they stood in dejected attitudes. On the 9th day Nos. 137 and 139 from time to time were seized with violent attacks of vomiting. The pupils of the eyes were dilated, tremors were frequent, and breathing was slow and labored. The other two birds also showed mild tremors and dyspnea. The following day Nos. 137 and 139 were in a paralyzed condition indistinguishable from the acute type of polyneuritis, except that the birds at times continued to show the symptoms of the previous day. No. 139 died in this condition on the 10th day. No. 137 was treated with autolyzed yeast filtrate and on the following day was less affected by paralysis but remained in a semicomatose condition for 5 days and died without further notable change. After 11 days on white rice Birds 138 and 140 also showed some paresis as indicated by drooping of the wings and a tendency to stumble in walking. The administration of α-hydroxypyridine to the birds was discontinued and both the remaining pigeons temporarily improved, although the diet of white rice was continued. Bird 138 died

on the 23rd day with typical symptoms of polyneuritis and No. 140 on the 40th day with mild chronic paralysis and in a condition of extreme weakness. The rate of loss of weight in all these birds did not differ materially from that commonly observed in pigeons fed on white rice alone.

Measurements were made daily of the rectal temperatures of these birds from the 8th to the 14th day. The observations are shown in Table I.

TABLE I.

Rectal Temperatures (°C.) of Birds Fed on White Rice ad libitum + 5 Mg. Daily of the Granular Form of α-Hydroxypyridine.

Day of experiment.	No. 137.	No. 138.	No. 139.	No. 140.	Average of 6 controls.
8th	40.1	41.3	40.8	42.1	42.1
9th	38.9	41.3	40.6	41.7	
10th	39.9*	40.1	Dead.	41.3	42.2
11th**	40.8	39.4		40.1	41.8
12th	39.9	39.6		40.1	41.7
13th	40.8	40.8		40.3	
14th	Dead.	41.2		41.3	42.0

* Became sick and was treated with autolyzed yeast.

** Hydroxypyridine discontinued.

It will be observed that the temperature fell from day to day as long as the treatment with α-hydroxypyridine was continued, but later gradually rose again in the case of the two birds which continued on white rice alone as well as Bird 137 which was treated with autolyzed yeast filtrate. A progressive fall of rectal temperature has been observed in scores of birds after being fed on white rice in this laboratory for 15 days or more. The extent to which the temperature drops is variable, being greatest when the development of polyneuritis is retarded, and is attended by much emaciation. Although the rectal temperature of healthy pigeons also is variable, under laboratory conditions it is not far from 42°C. In contrast, a number of birds in a condition of chronic polyneuritis have been found to show rectal temperatures from 34–35° after 30 to 50 days on white rice. However, the development of early and sudden severe attacks of polyneuritis in pigeons which are not markedly emaciated also often is attended by a rapid fall of rectal temperature. Following successful

treatment with α-hydroxypyridine or with autolyzed yeast the temperature often rises 1–2° in the course of a few hours, but has not been seen to go above normal. Such observations were first made nearly 2 years ago, but no opportunity has arisen for making a study of the causes of the phenomenon, although it would seem to be worthy of thorough investigation. In this connection attention is called to the rises above normal temperature following successful treatment of human beri-beri, reported by Williams and Saleeby (26).

Whatever the significance of the rise and fall of temperature, it will be noted that such a fall appears to be accentuated by administration of the granular form of α-hydroxypyridine. The experiment above described would seem to add weight to the impression previously recorded that the granular form of α-hydroxypyridine is more detrimental to polyneuritic pigeons than to healthy ones. In any case it seems certain that α-hydroxypyridine ultimately exerts a toxic action since symptoms develop which are not commonly found in polyneuritis.

α-Methoxypyridine.—α-Methoxypyridine was prepared from α-hydroxypyridine according to the directions of Pechmann and Baltzer (2). The substance conformed to the description of these authors. Polyneuritic pigeons (Nos. 43, 44, 45, and 46) were treated by intramuscular injection of 5, 2, 2, and 0.5 mg. of α-methoxypyridine in aqueous suspension. All four birds were rapidly prostrated and in each case within 20 minutes were apparently insensible. No. 43 died after 80 minutes, No. 44 after 35, and No. 45 after 75 minutes. After a few hours No. 46 recovered partly from the earlier prostration and lived for 24 hours, but without any relief of the paralytic symptoms.

α-Methylpyridone.—This substance was first prepared from α-hydroxypyridine by the method of Pechmann and Baltzer (2). A portion of the product as liberated from water solution by saturation with potassium carbonate was used for treating polyneuritic pigeons Nos. 61 and 62. No improvement resulted and the birds died the following night. The remainder of the product was dried over calcium oxide and distilled (boiling point 250°). Polyneuritic pigeon No. 64 received an injection of a freshly made water solution of 4 mg. of this preparation. After 2 hours the bird was relieved of neck retraction and was able to

walk a few steps. It maintained a constant weight for 3 days and gained markedly in strength and activeness. On the 4th day paralysis redeveloped and death ensued on the 5th day.

Unfortunately an accident resulted in the loss of the remainder of this preparation of methylpyridone. A second quantity was prepared, by the method of Decker (8), by the oxidation of pyridine methyl iodide with potassium ferricyanide. The oil was dried over calcium oxide and distilled (boiling point 250°). In Table II the freshly dehydrated product is designated as "dehydrated." The oil was kept in a cork-stoppered tube for 4 months awaiting a convenient opportunity for further tests. In Table II this product is designated as "hydrated" since it was subsequently found to have absorbed moisture. A portion was again dried over calcium oxide and redistilled. It was used fresh and is designated "redistilled" in Table II, which contains a summary of the results of the physiological tests. In all cases a drop of the oil was weighed into a dry tube. A measured amount of cold water was added, the solution taken up in a syringe, and an aliquot injected intramuscularly within a few moments.

As will be noted the number of cases in which improvement occurred in birds treated with freshly dehydrated methylpyridone was five, as against two apparent failures. Only one bird, No. 148, could be said to have shown a change of condition at all comparable to that produced by autolyzed yeast. The remainder though distinctly improved remained dejected. No substantial increases in weight were noted in any of the birds.

Nine birds were fed on white rice *ad libitum* and in addition each daily received by mouth 2 mg. of methylpyridone in the form of a freshly prepared water solution. After 20 days the administration of methylpyridone was discontinued and all the birds remaining at that time temporarily improved markedly in general condition. It was evident that the general health of the birds was adversely affected by the treatment. Five birds, of which two had typical symptoms of polyneuritis, died in 14, 26, 28, 28, and 30 days respectively. The remaining 4 were alive but extremely emaciated at the end of 40 days, when the experiment was discontinued.

TABLE II.

Action of α-Methylpyridone on Polyneuritic Pigeons.

Bird No.	Days fed on white rice before onset.	Symptoms.	Loss in weight.	Treatment.	Post-treatment condition.	Length of life after treatment.
			per cent			*days*
75	42	Mild chronic paralysis, very weak.	44	5 mg. "dehydrated."	No change.	3
76	29	Severe paralysis, neck retraction.	38	5 " "	Neck not retracted; could stand but not walk next day.	3
101	26	"	35	5 " "hydrated."	Dead next day.	
104	20	Severe paralysis, intermittent neck retraction.	27	5 " "	Slightly improved.	5
105	20	Severe paralysis.	25	5 " "	Worse.	1
108	42	Completely paralyzed, very weak.	42	5 " "	"	2
117B		Severe paralysis.		5 " "redistilled."	Distinct improvement for 2 days. Otherwise treated after 4 days.	
148	20	Severe paralysis, neck retraction.	20	5 " "	Complete recovery from paralysis in 2 days.	8

TABLE II—*Concluded.*

Bird No.	Days fed on white rice before onset.	Symptoms.	Loss in weight.	Treatment.	Post-treatment condition.	Length of life after treatment.
			per cent			*days*
173	30	Very weak and dejected, intermittent spastic attacks.	26	5 mg. "redistilled."	Free from spasticity next day; stronger and more vigorous on 2nd day.	6
200	24	Severe paralysis.	35	10 " "	Dead next day.	
207	24	"	34	10 " "	Intermittently spastic on 2nd day. Still further improved on 3rd day. Two doses were given on successive days.	4
				5 " "		

Trigonelline.—Nicotinic acid methyl iodide was shaken in water solution with silver oxide (24) and the resulting trigonelline hydrate was shaken out with chloroform and crystallized from alcohol and then from water. On drying at 100° the hydrate lost one molecule of water. The hydrate was administered to polyneuritic pigeons Nos. 78 and 79 without result. Seven birds (Nos. 80, 81, 113, 162, 172, 192, 197) received injections of 2 to 10 mg. of the dehydrated trigonelline, administration being effected under the same conditions as in the case of methylpyridone. It seems unnecessary to tabulate the results in detail. They may be summarized as follows: No. 80, after a dose of 3 mg. recovered completely from paralysis in the course of 10 hours, remained lively and active for 3 days, and died on the 7th. Nos.

162 and 172 improved markedly under similar conditions but lived only 3 days, without recovering complete muscular control. The remainder showed no improvement. There was no gain in weight in any of the individuals, though No. 80 maintained constant weight for 4 days.

Six birds were fed on white rice and given by mouth 5 mg. each daily of dehydrated trigonelline. The birds died with evidences of polyneuritis in 27, 34, 41, 41, 46, and 46 days respectively. Life may have been prolonged somewhat, but otherwise the course of the disease did not appear to be materially altered by the treatment.

Betaine.—A quantity of betaine hydrochloride (Kahlbaum's) was treated with sufficient sodium carbonate to combine with the hydrochloric acid present. The solution was evaporated to dryness, the residue taken up in absolute alcohol, and the resulting free betaine recrystallized from alcohol and dehydrated at 110°. Table III shows the results obtained by the expeditious injection of freshly prepared water solutions of the dehydrated betaine. Only in one case did treatment with dehydrated betaine fail to effect substantial improvement, and there is reason to suspect that repetition of the treatment would have led to successful results in this case.

Eight birds were fed on white rice *ad libitum*. On alternate days each received an injection of 5 mg. of betaine in the form of a freshly prepared water solution. Definite polyneuritic symptoms appeared in one bird after 21 days in the form of mild lameness, which varied greatly from day to day in severity. Death followed 15 days after the first appearance of the symptoms. A second bird died with typical symptoms on the 34th day, while a third and fourth were found dead on the 34th and 37th days without having shown definite neuritis. The remaining four birds remained alive at the end of 42 days, when the experiment was discontinued.

Nicotinic Acid.—Nicotinic acid was prepared by the oxidation of nicotine with nitric acid in the usual manner (27). Such preparations of nicotinic acid purified by recrystallization from water and alcohol have been administered to a score or more of polyneuritic pigeons and fowls during the past 2 or 3 years, but in no case was there indisputable evidence of benefit. Such a

TABLE III.

Action of Dehydrated Betaine on Polyneuritic Pigeons.

Bird No.	Days fed on white rice before onset.	Symptoms.	Loss in weight.	Treatment.		Post-treatment condition.	Length of life after treatment.
			per cent	*day*	*mg.*		*days*
147	47	Severe paralysis, neck retracted.	40	1st 2nd 3rd	5 5 5	Little change on 2nd; improved on 3rd day and held head normally; no further change.	5
149	16	"	27	1st 4th	5 5	No change for 3 days. Nearly free from paralysis on 5th day; able to fly fairly strongly on 6th.	11
165	14	"	36		3	Nearly recovered on 2nd day; able to fly strongly on 3rd and so continued till the 11th day.	18
179	25	"	36	1st 3rd	5 5	Free from paralysis but stupid on 2nd day; worse on 3rd; better again on 4th. Increasing weakness throughout.	5
181	26	"	32		5	Completely cured of paralysis on 2nd day. No symptoms till 9th day.	14
183	16	"	33		3	No change on 2nd day.	3
196	22	"	25		5	Free from symptoms on 2nd day; returned on 9th.	11
198	21	"	23	1st 2nd 3rd	5 5 5	Not improved on 2nd day; spastic only at intervals on 3rd; fully recovered on 4th; symptoms returned on 12th.	13
216	29	Moderate paralysis, severely prostrated.	26	1st 3rd	5 5	Gradual improvement till the 5th day but never quite free from paralysis. Decline apparent on 10th day.	13

TABLE III—*Concluded.*

Bird No.	Days fed on white rice before onset.	Symptoms.	Loss in weight.	Treatment.		Post-treatment condition.	Length of life after treatment.
			per cent	*day*	*mg.*		*days*
229	28	Severe paralysis.	40	1st 2nd 4th	5 5 5	Improvement marked on 2nd day and continued till the 6th; remained somewhat ataxic. Gained 12 gm. in weight.	9
235	25	"	26	1st 3rd	5 5	Improved on 2nd day; free from paralysis on 4th; able to run freely on 6th but could not fly; declining on 8th.	13

preparation of nicotinic acid was sublimed and 3 mg. of the sublimate were administered to polyneuritic pigeon No. 74 without result. Pigeon 199 received an injection of 3 mg. of nicotinic acid which had been continuously subjected to a temperature of 140° for 6 days. No improvement resulted. Pigeon 77 received without benefit 5 mg. of the substance after it had been heated in a sealed tube at 250° for 3 hours. The following day this bird was treated by another method. A small amount of nicotinic acid was converted into the hydrochloride. This was decomposed in water solution with freshly precipitated silver oxide, and the filtrate from the silver chloride was injected. Within 3 hours the bird was standing in a nearly normal attitude although it had been suffering from severe neck retraction when observed at frequent intervals for 24 hours previous. Nevertheless the pigeon was again completely paralyzed on the following day. It then received the filtered solution resulting from the decomposition by hydrogen sulfide of a few mg. of silver nicotinate suspended in water. No improvement ensued and the bird was dead the next morning. Pigeons 192 and 194 also were treated with the last mentioned product without result.

TABLE IV.

Effect on Polyneuritic Pigeons of Solutions Resulting from the Decomposition of Nicotinic Acid Hydriode with Silver Oxide.

Bird No.	Days fed on white rice before onset.	Symptoms.	Loss in weight.	Dose equivalent to nicotinic acid.	Post-treatment condition.	Length of life after treatment.
			per cent	*mg.*		*days*
143	32	Severe paralysis and neck retraction.	30	5	Assumed natural attitude after 3 hrs. Spastic at intervals. Still occasionally spastic next day. Free from paralysis on 2nd day but still weak. Weight constant for 5 days.	10
152	30	"	32	2	Distinct relief in 2 hrs. Little further improvement on 2nd day. Entirely free from paralysis on 3rd and able to fly. Gained 20 gm. in weight. Put on a diet of wheat on 7th day.	Still living and well.
155	33	General prostration.	46	5	Died during the night.	
193	16	Severe paralysis and neck retraction.	26	5	Nearly free from paralysis in 12 hrs. Able to fly strongly on 2nd day. Lively and vigorous. Gained 12 gm. in weight. Symptoms returned on 5th day.	5

Following Kirpal's method for converting nicotinic acid methyl iodide into trigonelline (24), nicotinic acid hydriodide was decomposed with silver oxide. Care was taken to avoid the use of any considerable excess of silver oxide. The fresh filtrate from the resulting silver iodide was injected promptly. Table IV shows the physiological results obtained. The rapidity with which the paralysis was relieved in Birds 143 and 152 is noteworthy.

SUMMARY.

1. The two crystalline forms of α-hydroxypyridine are isomers and mutually convertible into one another.

2. Only the needle form of this substance is curative for polyneuritis. The corresponding crystal form of β-hydroxypyridine and the anhydrous forms of methylpyridone, trigonelline, and betaine produce similar curative effects on polyneuritic birds. The last three named have been tested for protective properties with negative results.

3. It is concluded that the curative form of α-hydroxypyridine is a pseudo betaine and that a feature conforming more or less closely in structure or energy conditions to the type of a betaine ring is probably an essential characteristic of antineuritic "vitamines."

4. Attention is called to the theoretical possibility of the existence of such a structure in most of the simpler nitrogenous constituents of animal tissues, but especially in the nuclein bases.

6. Preliminary experiments are described which suggest that nicotinic acid may exist in a betaine form and that the curative properties of Funk's "vitamine" fractions of yeast and rice polishings may have been due in part to this isomeric form of nicotinic acid or a polymer or simple derivative of it.

BIBLIOGRAPHY.

1. Williams, R. R., *J. Biol. Chem.*, 1916, xxv, 437.
2. Pechmann, H. v., and Baltzer, O., *Ber. chem. Ges.*, 1891, xxiv, 3144.
3. Decker, H., *J. prakt. Chem.*, 1900, lxii, 266.
4. Kauffmann, H., *Ber. chem. Ges.*, 1903, xxxvi, 1062.

5. Koenigs, W., and Koerner, G., *Ber. chem. Ges.*, 1883, xvi, 2160. Koenigs, W., and Geigy, R., *ibid.*, 1884, xvii, 589. Pechmann, H. v., and Welsh, W., *ibid.*, 1884, xvii, 2390. Feer, A., and Koenigs, W., *ibid.*, 1886, xix, 2432. Fischer, O., *ibid.*, 1898, xxxi, 609; 1899, xxxii, 1297. Gutbier, A., *ibid.*, 1900, xxxiii, 3358. Pechmann, H. v., *ibid.*, 1895, xxviii, 1624. Weidel, H., and Strache, H., *Monatsh. Chem.*, 1886, vii, 297. See also preceding references 2, 3, and 4.
6. Fischer, O., and Renouf, E., *Ber. chem. Ges.*, 1884, xvii, 763.
7. Weidel, H., and Blau, F., *Monatsh. Chem.*, 1885, vi, 664. Fischer and Renouf, *Ber. chem. Ges.*, 1884, xvii, 1896.
8. Decker, *J. prakt. Chem.*, 1893, xlvii, 28.
9. Willstätter, R., *Ber. chem. Ges.*, 1902, xxxv, 584.
10. Haitinger, L., and Lieben, A., *Monatsh. Chem.*, 1885, vi, 298. Meyer, H., *ibid.*, 1907, xxviii, 47.
11. Williams, unpublished work.
12. Drummond, J. C., and Funk, C., *Biochem. J.*, 1914, viii, 612.
13. Barger, G., The Simpler Natural Bases, London, 1914, p. 40.
14. Funk, C., *J. Physiol.*, 1912–13, xlv, 489.
15. Williams, R. R., and Seidell, A., *J. Biol. Chem.*, 1916, xxvi, 431.
16. Meyer, *Monatsh. Chem.*, 1902, xxiii, 942; 1903, xxiv, 195; 1904, xxv, 491; 1905, xxvi, 1303; 1906, xxvii, 31, 255, 987; 1907, xxviii, 33, 47.
17. Geake, A., and Nierenstein, M., *Biochem. J.*, 1915, ix, 309.
18. Griess, P., *Ber. chem. Ges.*, 1880, xiii, 246.
19. Engler, C., and Bauer, F. W., *Ber. chem. Ges.*, 1894, xxvii, 1775. Engler, C., and Kronstein, A., *ibid.*, 1894, xxvii, 1779.
20. Warmington, E., *J. prakt. Chem.*, 1893, xlvii, 214. Johnson, T. B., and Hill, A. J., *J. Am. Chem. Soc.*, 1914, xxxvi, 1201.
21. Vedder, E. B., and Williams, R. R., *Philippine J. Sc., B*, 1913, viii, 183.
22. Funk, *J. Physiol.*, 1913, xlvi, 177.
23. Turnau, R., *Monatsh. Chem.*, 1905, xxvi, 537.
24. Kirpal, A., *Monatsh. Chem.*, 1901, xxii, 365.
25. Koenigs and Geigy, *Ber. chem. Ges.*, 1884, xvii, 589.
26. Williams, R. R., and Saleeby, N. M., *Philippine J. Sc., B*, 1915, x, 99.
27. Pictet, A., and Sussdorff, G., *Arch. Sc. phys. nat. Genève*, 1898, Series 4, v, 113.

EXPLANATION OF PLATE 4.

FIG. 1. A microscopic field of the freshly prepared needle form of α-hydroxypyridine contaminated with a few granular crystals, the material being mounted in a benzene-ligroin mixture.

FIG. 2. The same microscopic field reproduced as it appeared after the lapse of 30 minutes. Note the growth of the granular form at points of original contamination. Further transformation has been retarded by the spontaneous evaporation of the film of liquid, the receding edge of which is seen in the upper photograph.

FIG. 1.

FIG. 2.

(Williams: Chemical Nature of Vitamines.)

THE DIETARY DEFICIENCIES OF THE WHITE BEAN, PHASEOLUS VULGARIS.*

By E. V. McCOLLUM, N. SIMMONDS, AND W. PITZ.

(*From the Laboratory of Agricultural Chemistry, University of Wisconsin, Madison.*)

PLATE 5.

(Received for publication, February 27, 1917.)

Preliminary to our plan for making an elaborate inquiry into the supplementary dietary relationships among our natural foodstuffs, we have carried out a series of feeding experiments with a long list of the individual natural foods. We have supplemented these with single and multiple additions of purified food factors, *e.g.*, protein, inorganic salt mixtures of appropriate composition, fat-soluble A, and water-soluble B, in order to evaluate these several dietary factors in each of the natural products which we are studying in combinations. We have described in previous papers the results obtained by this procedure with wheat,[1] rice,[2] wheat germ,[3] maize,[4] and oat kernel.[5]

In the present paper we describe the behavior of young rats when fed the white bean of commerce as the principal constituent of the diet, and supplemented with purified food factors in various ways. The results indicate clearly the low biologic value

* Published with the permission of the Director of the Wisconsin Experiment Station.

[1] Hart, E. B., and McCollum, E. V., *J. Biol. Chem.*, 1914, xix, 373.
McCollum, E. V., and Davis, M., *ibid.*, 1915, xxi, 615.
Hart, E. B., Miller, W. S., and McCollum, E. V., *ibid.*, 1916, xxv, 239.
McCollum, E. V., Simmonds, N., and Pitz, W., *ibid.*, 1916–17, xxviii, 211.

[2] McCollum and Davis, *J. Biol. Chem.*, 1915, xxiii, 181.

[3] McCollum, Simmonds, and Pitz, *J. Biol. Chem.*, 1916, xxv, 105.

[4] McCollum, Simmonds, and Pitz, *J. Biol. Chem.*, 1916–17, xxviii, 153.

[5] McCollum, Simmonds, and Pitz, *J. Biol. Chem.*, 1917, xxix, 341.

of the proteins of the bean when fed as the sole source of amino-acids.

Because of the high content of protein in beans, they, or more generally the legumes as a class, have been frequently recommended as substitutes for the apparently more expensive sources of protein; *viz.*, meat, milk, and eggs. It is clearly apparent that such advice may be misleading, since modern investigations of proteins by chemical and biologic methods have made it clear that from the dietetic standpoint the term protein cannot be loosely employed as it was formerly. Protein is of biologic value only in so far as it yields appropriate proportions of the essential amino-acids. The facts brought out by the data here presented may be summarized as follows.

1. The low nutritive value of the bean proteins is shown by the behavior of Lot 718 (Chart 1) whose ration contained 19.8 per cent of proteins all derived from the bean. There was high mortality in animals fed this ration, and pronounced stunting was observed in all.

Lot 830 (Chart 2) whose ration contained 19.0 per cent of protein from the bean and 3 per cent of casein was noticeably improved as the sole result of the small casein addition. It is evident from the results shown in Charts 1 and 2 that there is only a moderately efficient supplementing action of bean proteins by casein. The improvement in Lot 830 over Lot 718 seems to be due to the *additional* protein as casein in greater measure than to a marked increase in the value of the beans through the supplying of a particular amino-acid present in very small amount in bean proteins and plentiful in casein.

Lot 720 (Chart 3) and Lot 721 (Chart 4) serve further to support the view expressed above regarding the low biologic value of the bean proteins. Our experience with the group of proteins contained in the bean confirms the experience of Osborne and Mendel[6] with phaseolin, the principal protein of the bean, which they isolated and fed in purified form supplemented with "protein-free milk" as a source of the unidentified dietary factors and inorganic salts. They found phaseolin to possess very little biologic value.

[6] Osborne, T. B., and Mendel, L. B., *Z. physiol. Chem.*, 1912, lxxx, 307.

2. A high content of beans in the diet appears to exert an injurious effect on the rat. This is illustrated by the history of Lot 720 (Chart 3) as compared with that of Lot 621 (Chart 5). The only significant difference in the rations of these two groups was in the content of beans. The former contained 70 per cent of beans and the latter 45 per cent. Each ration contained 9 per cent of casein. The animals fed the lower content of beans (Lot 621) and consequently the lower protein supply were much better nourished than were those in Lot 720. The same is true of Lot 719 (Chart 6) with a ration containing 50 per cent of beans with 4 per cent of casein as contrasted with Lot 830 (Chart 2) whose ration contained 86.3 per cent of beans and 3 per cent of casein. The latter with 19 per cent of protein were not so well nourished as the former with but 15 per cent. The young which were successfully weaned in Lot 621, with the lower content of beans, were able to grow normally to full adult size and produce young. Those which were reared on Ration 720 with the higher bean content were able to grow at less than half the normal rate (compare Charts 3 and 5). Two explanations seem sufficiently plausible to warrant brief comment. It may be that beans contain some unknown chemical complex which is harmful and that young rats can tolerate without pronounced injury the intake of the hypothetical substance carried by the lower plane of intake of beans, but show the effects of intensifying this factor. Provisionally, however, we have adopted the view that the rats are mechanically injured by the distention of the digestive tract, due to the extent of fermentation processes after eating liberally of beans. The latter contain much carbohydrate of the class of the hemicelluloses, for the digestion of which the higher animals have no provision. These hemicelluloses are, however, readily attacked by microorganisms inhabiting the digestive tract, and their fermentative decomposition is accompanied by the liberation of a great deal of gas. That this is in great measure responsible for the injurious effects following high bean feeding is supported by the marked tympanites observed regularly in experimental animals eating rations high in beans (Figs. 1 and 2). In rats under 45 gm. body weight the distention of the intestinal tract with gas appears to be the direct cause of death on such rations. Partial asphyxiation of

the walls of the intestinal tract through interferences of the circulation caused by excessive stretching is, we believe, the principal factor in debilitating the animals, but the low quality of the proteins is in some of the rations here described a contributing factor.

3. The white bean appears to be even poorer in its content of the dietary factor fat-soluble A than are the cereal grains. This is illustrated by the records of Lot 611 (Chart 7) whose ration in Period 1 was closely similar to that of Lot 621 (Chart 5), except that no butter fat was included. During a period of 8 weeks there was no growth on this ration, but the animals responded at once with growth at a good rate when in the second period 5 per cent of butter fat replaced its equivalent of beans.

4. The inorganic content of the bean is not of a character which supports growth. This is seen in the records of Lot 778 (Chart 8) whose ration closely resembled that of Lot 621 (Chart 5) except that no salt mixture was added in Period 1. No growth took place until in the second period 1.90 per cent of a suitably constituted salt mixture was introduced, when the animals at once responded with increase in body weight. In this respect the bean behaves like all other seeds which we have studied. In all cases the seeds of plants require a modification of their inorganic content by salt additions before growth can take place when the other dietary factors are suitably improved by the addition of purified food substances. With combinations of seed with leaf we have been able to nourish rats adequately through four generations on a strictly vegetarian diet without either protein, salt, or fat-soluble A additions.[7] This is possible because of the high content and peculiar composition of the inorganic content of the leaf[8] and its richness in the fat-soluble A.

5. The richness of the bean in the unidentified water-soluble B is shown by the remarkable history of Lot 696 (Chart 9) whose ration consisted of a mixture of purified foodstuffs including 5 per cent of butter fat to furnish the fat-soluble A. The ration was so composed as to require only the addition of the water-soluble B to support growth. The sole function of the 25 per cent of beans in the food mixture was to furnish this dietary factor. On this ration growth was complete and one female has produced two

[7] McCollum, Simmonds, and Pitz, *Am. J. Physiol.*, 1916, xli, 333.

[8] Forbes, E. B., *Ohio Agric. Exp. Station Bull. 222*, 1910.

litters of young (eighteen young) of which eight were reared. The beans employed in this ration were soaked in distilled water and heated for $1\frac{1}{4}$ hours at 15 pounds' pressure, dried, and ground.

Mendel and Fine[9] noted the relatively low utilization of the proteins of the bean and showed that the cause lay in part in the high content of indigestible carbohydrate. This prevents digestion to some extent by protecting the proteins from contact with the digestive juices. The indigestible carbohydrate likewise acts as a sponge to absorb the secretions of the tract with their content of proteins and their cleavage products, and prevents their reabsorption.[10] Our own experiments furnish conclusive evidence that the entire protein mixture of the bean is of low value, but further inquiry must be made to show which amino-acid is the limiting factor in determining the poor quality of these proteins.

Goldberger[11] has emphasized the desirability, in planning dietaries for the treatment of pellagra, or for the prevention of its recurrence, of including fresh animal and leguminous protein foods. It is, of course, probable that properly planned experimental inquiry will show that when combined with appropriate proportions of the protein of certain other natural foods, the proteins of the bean may be greatly enhanced in biologic value. We have under way an investigation of this question. It should be emphasized that the evidence at present available points to the conclusion that the bean proteins are decidedly inferior to those of meat, milk, eggs, and the cereal grains which have thus far been carefully studied, *viz.*, wheat, maize, oat, and rice kernels.

The bean contains an abundance of the unidentified water-soluble B, since 25 per cent of beans as the sole source of this factor (Chart 9) supports completion of growth and repetition of reproduction. In this respect it does not differ markedly from the cereal grains.[12] We would point out, therefore, that by the methods of investigation we have employed, no peculiar dietary properties of the bean indicating unusual value have

[9] Mendel, L. B., and Fine, M. S., *J. Biol. Chem.*, 1911–12, x, 433.
[10] Mendel, L. B., and Lewis, R. C., *J. Biol. Chem.*, 1913–14, xvi, 55.
[11] Goldberger, J., *J. Am. Med. Assn.*, 1916, lxvi, 471.
[12] McCollum, Simmonds, and Pitz, *J. Biol. Chem.*, 1916–17, xxviii, 229.

been revealed. Unless, as has been pointed out above, it shall be found that proteins from other constituents of the diet make good the chemical deficiencies of the proteins of the bean, the generalization of McKay[13] that they (the legumes) "as a source of protein are superior to most of the cereals" cannot be accepted in the light of experimental work now available. Judging from the decided damage to the intestine which results from the excessive fermentation of the carbohydrates of beans we feel safe in saying that this legume should under no circumstances form a principal part in any relatively monotonous diet such as many institutions even in this country furnish their inmates.

Finally, mention should again be made of the fact that our simple interpretation of the causes of failure in the case of certain of our diets proves adequate in all cases. The data furnished in this and our former papers[1–5] now warrant the conclusion that the great problem in practical dietetics and in animal production lies not in securing enough of the unidentified chemical complex associated with the causation of polyneuritis (water-soluble B), but in securing a satisfactory adjustment among (1) the digestion products of the proteins of the diet (amino-acids), (2) an adequate supply of the fat-soluble A, and (3) more important even than a high biologic value of the protein mixture of the ration, a content of the essential inorganic elements suitable in amounts and proportions.

Our extensive experience in feeding natural food substances restricted to a single source now leaves no room for doubt that the character of the inorganic content of the food mixture if fed monotonously constitutes a factor of the greatest importance in influencing growth and well-being. Furthermore, it is now evident that so small an amount as 15 per cent of wheat kernel, 3 per cent of wheat germ, or 25 per cent of thoroughly cooked beans serve to supply an ample amount of all the as yet chemically unidentified food essentials except that supplied by butter fat (fat-soluble A). We are convinced that the prevalent tendency to regard several types of malnutrition (scurvy, pellagra, rickets, etc.) as being each due to the lack of a specific chemical complex in the diet cannot be supported by convincing experimental proof. Any diet containing a moderate content of a wholesome natural food will not be lacking in the water-soluble B.

[13] McKay, D., The Protein Element in Nutrition, London, 1912, p. 58.

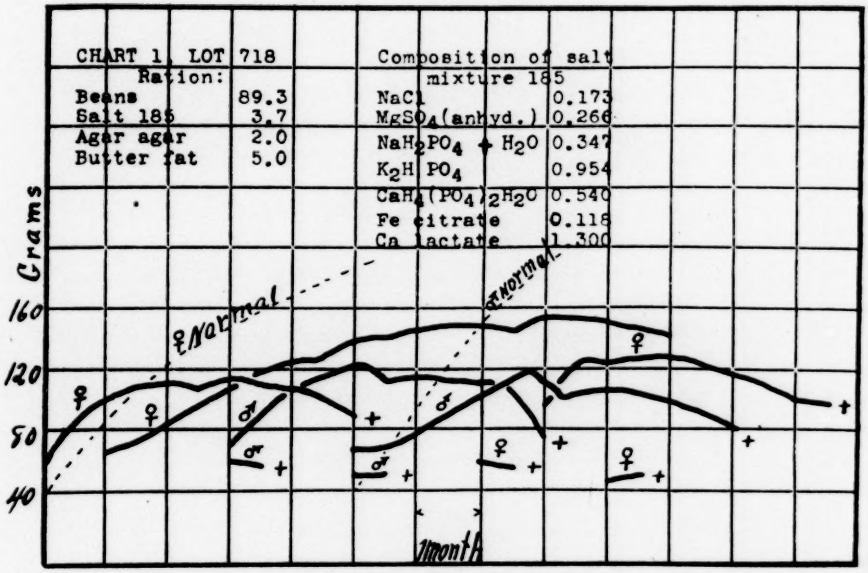


CHART 1. Lot 718 illustrates the poor growth of rats fed a diet which as far as concerns chemical complexes is an adequate food mixture except for the poor quality of the proteins, all of which (19.6 per cent) are derived from beans. The validity of this explanation is evident from the records of Lot 830, Chart 2, and of Lot 720, Chart 3, whose rations were closely similar to those of Lot 718, Chart 1, except that 3 per cent and 9 per cent of casein respectively were included in the diets. Four younger animals failed entirely to grow on this diet and died after 2 to 3 weeks. We attribute a positive injurious effect of a high bean content in a monotonous diet to the excessive fermentation of hemicelluloses.

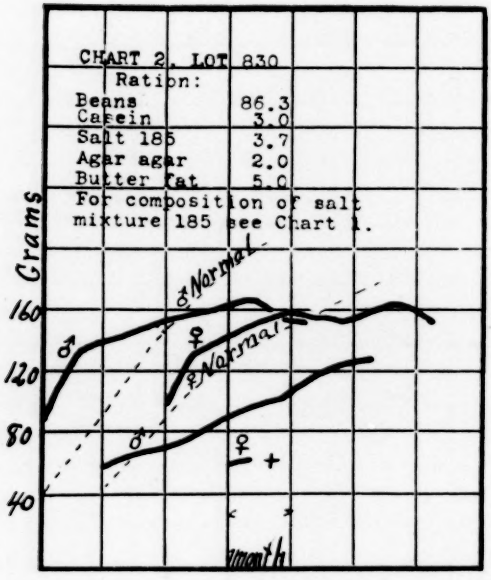


CHART 2. Lot 830 shows that 3 per cent of casein superimposed upon 19 per cent of bean proteins does not greatly increase the value of the latter. This we interpret as an indication that the supplementary relationship of casein for the bean proteins is not exceptionally efficient in the sense that casein is very rich in the one or more amino-acids which are present in smallest amounts and therefore form the limiting factors which determine the biologic value of the bean proteins. Owing to the depressing effect of the excessive gas formation which may in some degree mask a moderate improvement in the quality of the protein through small casein additions, we do not feel that with the data at present available this point can be settled with certainty. This chart should be compared with Charts 1 and 3.

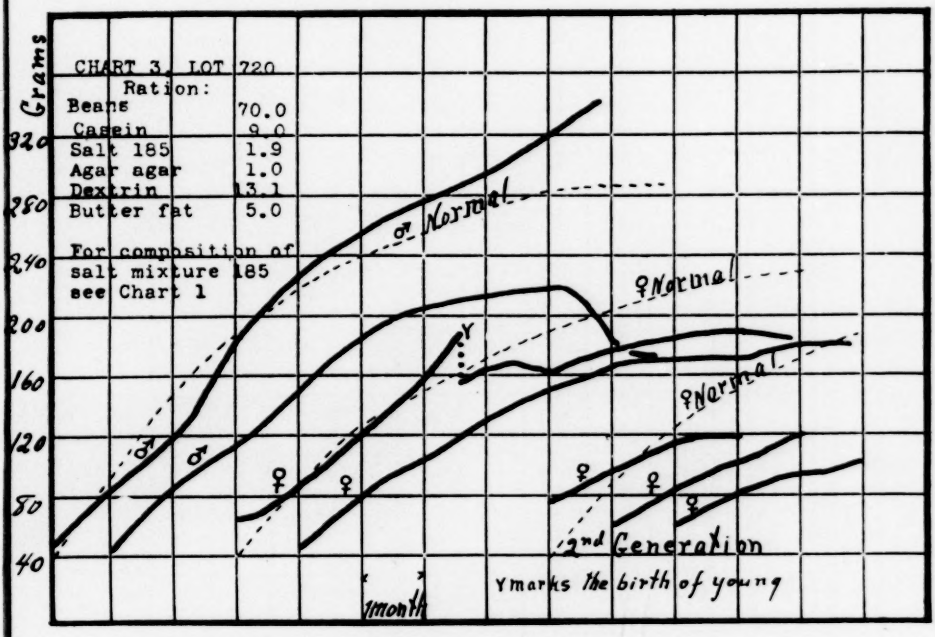


CHART 3. Lot 720 shows the growth obtained in rats fed a ration closely similar to those of Charts 1 and 2 but with a higher content of casein (9 per cent) supplementing the bean proteins (16.94 per cent) of the food mixture. With this amount of casein normal growth was secured. These records when considered in connection with those of Charts 1 and 2 establish the fact that the proteins of the bean are of very low biologic value and they must be combined with proteins from other sources which are so constituted as to make good their deficiencies with respect to certain amino-acids before they can become efficient for supporting growth. The decided improvement in the growth of rats receiving bean proteins with very small casein additions (3 to 4 per cent) (Charts 2 and 6) supports the belief that casein does supply somewhat liberally certain amino-acids in which the bean proteins are especially poor. The disturbing influence of the excessive fermentation in the intestine in animals fed such rations as are described in these charts makes it impossible at present to pass definitely upon this point. This ration does not support optimum well-being in the animals, however, for the two females of this group produced but a single litter of young up to the age of 9 months and these young, continued on the mother's ration, grew only at about half the normal rate. We are inclined to attribute this debilitating effect in part at least to mechanical injury to the digestive tract.

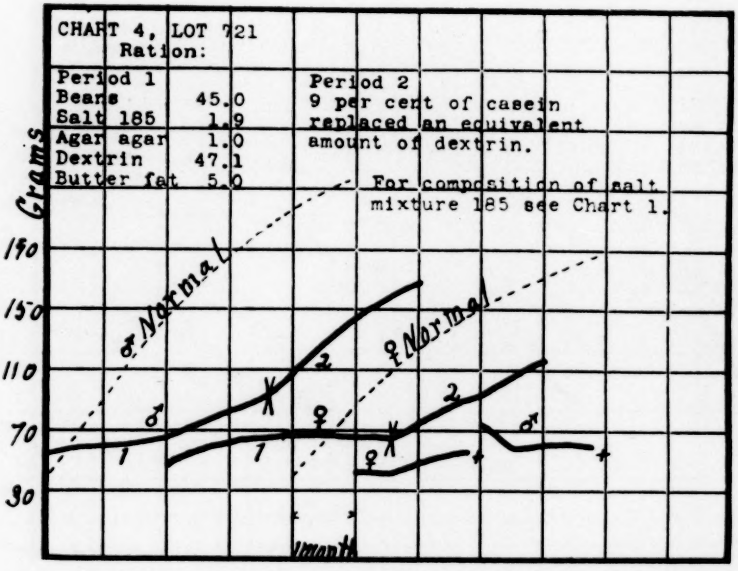


CHART 4. Lot 721 was introduced into the series of experiments here described to ascertain whether by reducing the content of beans, and therefore the amount of indigestible but fermentable carbohydrates (hemicelluloses), the animals might make better use of the proteins of the bean. This did not prove to be the case. After 3½ months of suspended growth they responded promptly when 9 per cent of casein was introduced into the diet. Compare these records with those of Lot 621, Chart 5, whose diet contained 9 per cent of casein throughout the experiment but was otherwise identical with that of Lot 721.

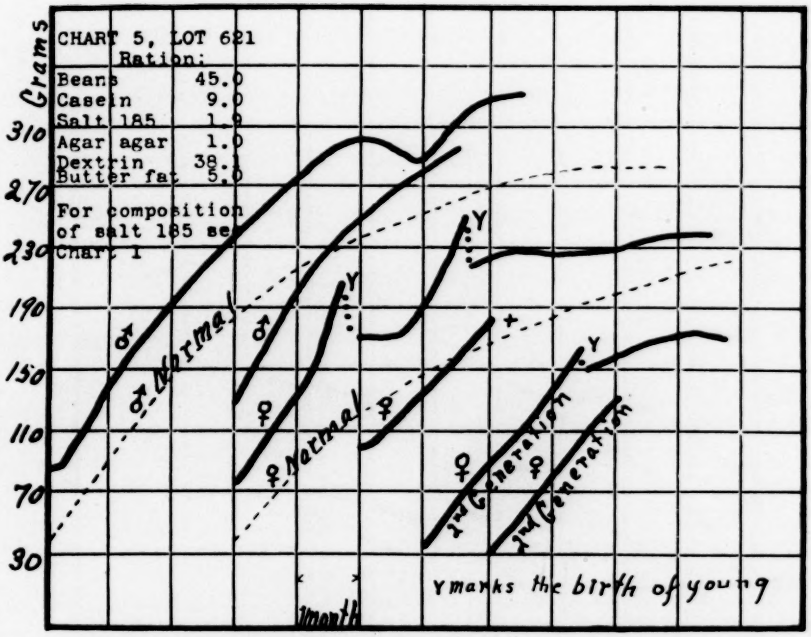


CHART 5. Lot 621. These records, when considered along with those of Chart 4, Lot 721, indicate clearly that the factor which is responsible for the poor nutrition of the latter was the poor quality of the proteins of the beans. That the content of the beans in easily fermentable hemicelluloses, for the digestion of which the higher animals are not provided with enzymes, is a depressing factor in our experiments containing beans is emphasized by a comparison of Chart 5 with Chart 3, Lot 720, whose ration was closely similar but contained 70 per cent of beans. The rats receiving the lower content of total protein (and hemicellulose) were distinctly better nourished than those receiving the higher bean allowance. This we attribute to the mechanical injury resulting from distention of the digestive tract with gas. Ration 621 contained 18.9 per cent, and Ration 720 24.4 per cent of protein.

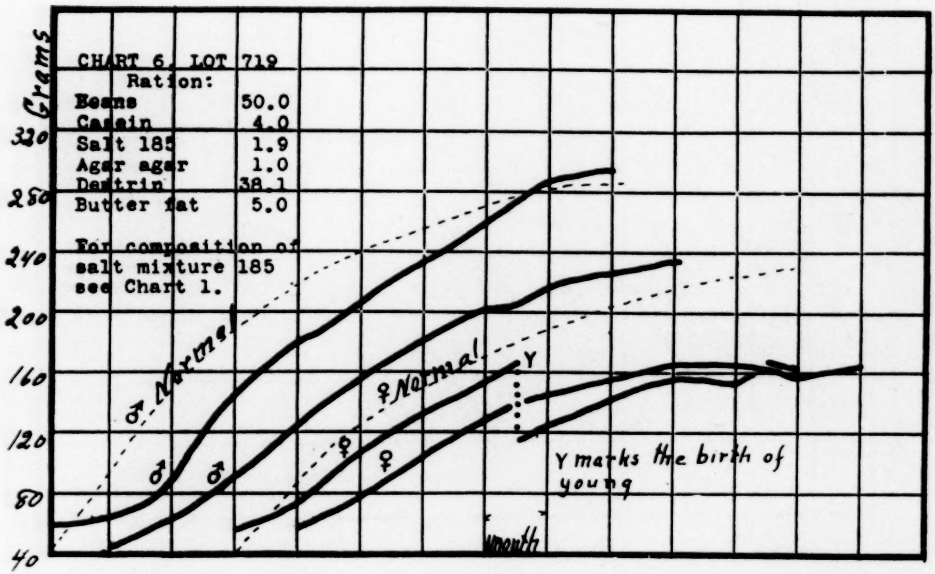


CHART 6. Lot 719 illustrates the character of the growth curves obtained with a diet all factors of which were fairly satisfactorily constituted but which derived its protein (15 per cent) from 50 per cent of beans and 4 per cent of casein. Growth was somewhat below normal and reproduction greatly depressed. No young were reared. Poor quality of the protein moiety and the depressing effects of excessive intestinal fermentation are the causes of the unsatisfactory nutrition of these rats. Compare with Lot 830, Chart 2, and Lot 621, Chart 5.

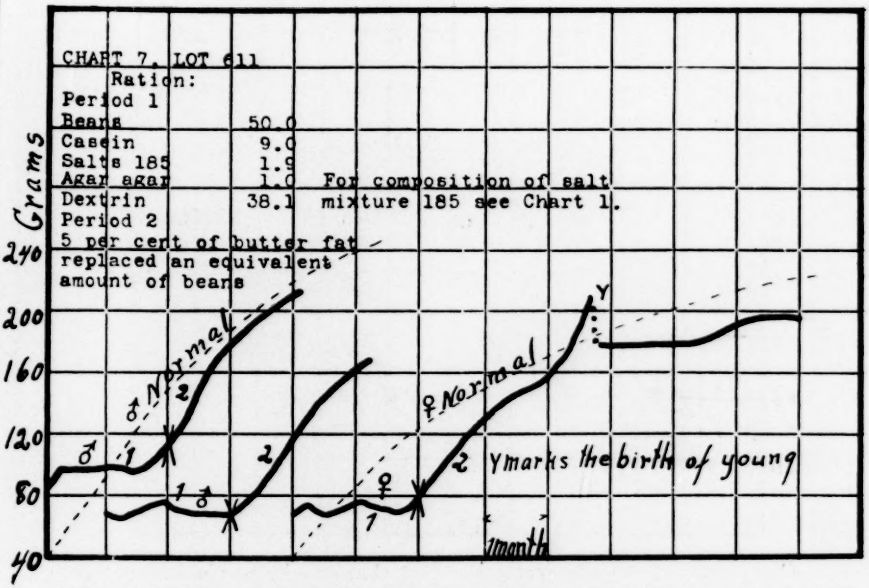


CHART 7. Lot 611 shows that beans contain very little, if any, of the unidentified fat-soluble A. In Period 1 there was no growth, although the diet was adequate except for its lack of the fat-soluble A. After 2 months' suspension of growth the animals responded with normal growth on the inclusion in Period 2 of 5 per cent of butter fat in the diet. This diet contained 20 per cent of protein, 11 per cent of which was furnished by beans.

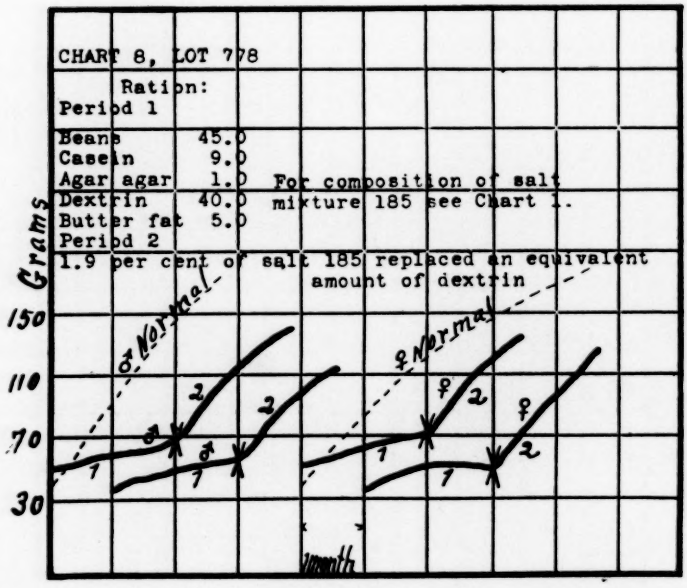


CHART 8. Lot 778 illustrates the fact that the inorganic content of 45 per cent of beans, supplemented with the phosphorus and sulfur in 9 per cent of casein does not support growth even though the organic portion of the ration is adequate for fairly good nutrition over a long period (compare Chart 5, Lot 621). In Period 2 the addition of 1.9 per cent of Salt Mixture 185 induced prompt resumption of growth at the normal rate. The bean, like the cereal grains we have studied,[1–5] contains an inorganic content which is unsatisfactory in its content and composition for the promotion of physiologic well-being in a growing animal.

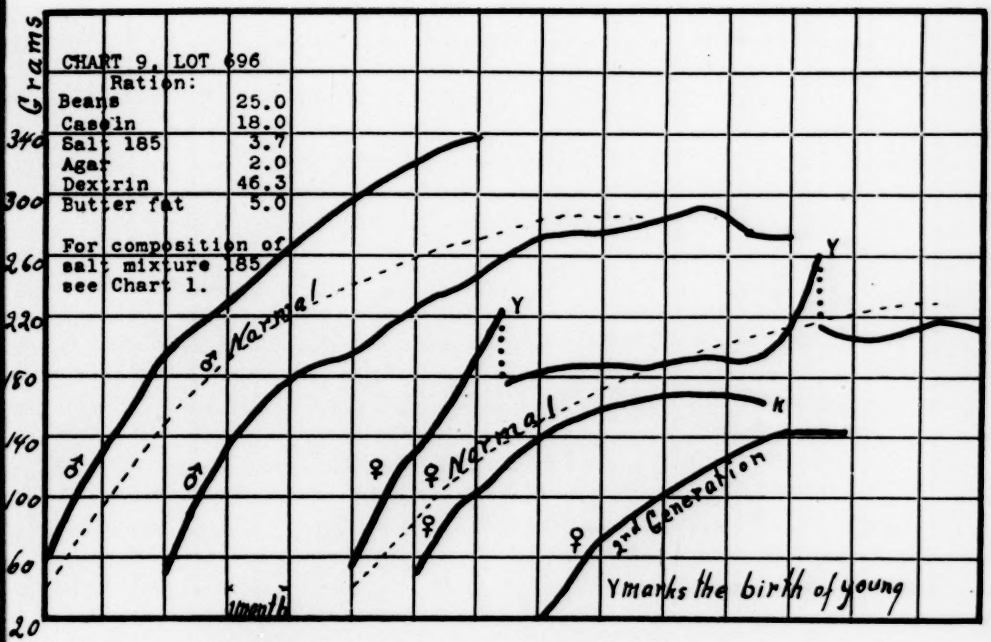


CHART 9. Lot 696 shows the richness of the bean in the unidentified dietary factor water-soluble B. This ration, aside from its bean content, consists of a mixture of purified food substances which supplies adequate protein, salts, carbohydrate, and fat-soluble A (in the butter fat). The sole essential function of the 25 per cent of beans in the food mixture is to furnish the water-soluble B. The beans were soaked in water and then heated for 1¼ hours in an autoclave at 15 pounds' pressure, dried, and ground before incorporation with the ration. Even after this drastic treatment, beans to the extent of one-fourth of the food mixture supplied enough of the factor B to induce perfectly normal growth. One female produced two litters of young (eighteen young), of which eight were successfully weaned. The curve of one of her daughters is practically normal on this ration.

EXPLANATION OF PLATE 5.

FIG. 1. The appearance of the abdomen of a normal rat.

FIG. 2. The appearance of the abdomen of a rat fed a monotonous ration containing a high content of beans. Note the marked tympanites.

FIG. 1.

FIG. 2.

(McCollum, Simmonds, and Pitz: Deficiencies of White Bean.)

INDEX TO VOLUME XXIX.

ACID, amino-, nitrogen content of blood of various species, 191
—— butyric, in biological products, 199
—— hydrocyanic, in *Sorghum vulgare*, 25
——, oleic, and sodium bicarbonate, bile and bile salts in reaction of, 367
——, thiobarbituric, as qualitative reagent for ketohexose, 207
——, yeast nucleic, nucleotide linkage in, 123
——, —— ——, structure of, 111
Acid-base-producing properties of diet in relation to alkali administration and blood sugar, 227
Acid-producing diet, influence of, on blood sugar, 233
ADDIS, T., and WATANABE, C. K. The rate of urea excretion. III. The effect of changes in blood urea concentration on the rate of urea excretion, 391. IV. The effect of changes in the volume of urine on the rate of urea excretion, 399
Adenine-uracil dinucleotide, 111
Adipocere, 319
ADOLPH, E. F. See WILSON and ADOLPH, 405
Alkali administration, influence of, on blood sugar, 227
Amino-acid nitrogen content of blood of various species, 191
Ammonia, micro-titration of, 459
—— in urine, new reagent for separation of, 329
Anesthetics, effect on cyanogenetic compounds of *Sorghum vulgare*, 37
Animal diastases, 179
Ash and protein for growing animals, corn as source of, 485
Athyrosis, fetal, 215
Autolysis, bile in, 281

BARNETT, G. D. The micro-titration of ammonia, with some observations of normal human blood, 459
Base- and acid-producing properties of diet in relation to alkali administration and blood sugar, 227
Base-producing diet, influence on blood sugar, 233
Bean, white, dietary deficiencies of, 521
BELL, R. D. See FOLIN and BELL, 329
Bence-Jones proteinuria, 425
BERGEIM, O. See HALVERSON and BERGEIM, 337
Bile in autolysis, 281
—— derivatives in Bloor's cholesterol determination, 463
—— in reaction of oleic acid and sodium bicarbonate, 367
Biological products, butyric acid in, 199
Blood, amino-acid nitrogen content of, of various species, 191
——, determination of cholesterol in, 437
—— of fresh water fish, partition of non-protein nitrogen in, 405
——, human, cholesterol and its esters in, 7
——, ——, —— in, under pathological conditions, 93

Blood, human, factors involving accuracy of creatinine determinations in, 47
——, increased diastatic activity in diabetes and nephritis, 179
—— and milk, rapid method for determining calcium in, 169
——, normal human, micro-titration of ammonia in, 459
—— and plasma, creatine and creatinine in, 413
——, sugar content of, and epinephrine hyperglycemia and glycosuria, influence of intravenous injection of Witte's peptone upon, 127
—— ——, influence of acid-base-producing diet on, 233
—— ——, —— —— alkali administration on, 227
—— urea concentration, effect on rate of urea excretion, 391
Bloor, W. R. The determination of cholesterol in blood, 437
—— and Knudson, A. Cholesterol and cholesterol esters in human blood, 7
Bock, J. C. The amino-acid nitrogen content of the blood of various species, 191
Bradley, H. C., and Taylor, J. Studies of autolysis. V. The influence of bile on autolysis, 281
Buckner, G. D., and Kastle, J. H. The growth of isolated plant embryos, 209
Butyric acid in biological products, 199

Calcium in blood and milk, rapid method for determining, 169
—— content of cerebrospinal fluid, 337
Carbohydrate metabolism, 227, 233, 245, 251, 255, 265, 273
Cereal grains, chickens on rations restricted to, 57
Cerebrospinal fluid, calcium content, 337
Chemical composition of placenta, 19
—— nature of the vitamines, 495
Cholesterol in blood, determination of, 437
—— and cholesterol esters in human blood, 7
—— determination, Bloor's, bile derivatives in, 463
—— in human blood under pathological conditions, 93
Colorimeter-nephelometer, 155
Colorimetric determination of ammonia in urine, 329
Corn gluten, relative value of certain proteins and protein concentrates as supplements to, 69
—— as source of protein and ash for growing animals, 485
Corner, G. W. Variation in the amount of phosphatides in the corpus luteum of the sow during pregnancy, 141
Corpus luteum of sow, variation of phosphatides in, during pregnancy, 141
Cotton seed as food, 289
Creatine and creatinine in whole blood and plasma, 413
—— excretion, diurnal variations in, 447
——parenterally introduced, excretion of, 1
Creatinine and creatine in whole blood and plasma, 413
—— determinations in human blood, factors involving accuracy of, 47
—— parenterally introduced, excretion of, 1
Cyanogenetic compounds of *Sorghum vulgare*, effect on, of anesthetics and frosting, 37

DENIS, W. Cholesterol in human blood under pathological conditions, 93
——. A note on the diurnal variations in creatine excretion, 447
Determination of ammonia in urine, colorimetric, 329
—— —— butyric acid in biological products, 199
—— —— calcium in blood and milk, rapid method, 169
—— —— cholesterol in blood, 437
—— —— ——, Bloor's, bile derivatives in, 463
—— —— creatinine in human blood, factors involving accuracy of, 47
—— —— hydrocyanic acid in *Sorghum vulgare*, 25
—— —— permeability, direct, 453
—— —— total sulfur in urine, note on Benedict's method for, 15
Diabetes and nephritis, increased diastatic activity of blood in, 179
Diastases, animal, 179
Diastatic activity of the blood, increased, in diabetes and nephritis, 179
Diet, acid-base-producing, influence of, on blood sugar, 233
——, —— properties, in relation to alkali administration and blood sugar, 227
——, cotton seed, 289
—— and glycogen content of liver, 255
——, relation to epinephrine glycosuria, 245
Dietary deficiencies of oat kernel, 341
—— —— —— white bean, 521
Dinucleotide, adenine-uracil, 111

EMBRYOS of plants, isolated, growth of, 209
Epinephrine glycosuria, relation of dosage and diet to, 245
Epinephrine hyperglycemia and glycosuria, influence of sodium carbonate, 251
—— —— —— ——, and sugar content of blood, influence of intravenous injection of Witte's peptone upon, 127
Esters of cholesterol in human blood, 7

FENGER, F. The chemical composition of the placenta, 19
Fetal athyrosis, 215
FOLIN, O., and BELL, R. D. Applications of a new reagent for the separation of ammonia. I. The colorimetric determination of ammonia in urine, 329
Frosting, effect of, on cyanogenetic compounds of *Sorghum vulgare*, 37

GETTLER, A. O. Factors involving the accuracy of creatinine determinations in human blood, 47
GIVENS, M. H. A note on Benedict's method for the estimation of total sulfur in urine, 15
Glucose administration, hyperglycemia and glycosuria after, influence of sodium carbonate, 265
Gluten of corn, relative value of certain proteins and protein concentrates as supplements to, 69
Glycogen content of liver, relation to diet, 255
Glycosuria, epinephrine, relation of dosage and diet to, 245
—— and hyperglycemia, epinephrine, influence of sodium carbonate, 251
—— —— ——, ——, and sugar content of blood, influence of intravenous injection of Witte's peptone upon, 127

Glycosuria and hyperglycemia after glucose administration, influence of sodium carbonate, 265
——, salt, mechanism, 273
Grains, cereal, chickens on rations restricted to, 57

HALPIN, J. G. See HART, HALPIN, and MCCOLLUM, 57
HALVERSON, J. O., and BERGEIM, O. The calcium content of cerebrospinal fluid, particularly in tabes dorsalis, 337
HAMMETT, F. S. Variations in the composition of human milk during the first 11 days after parturition, 381
HART, E. B., HALPIN, J. G., and MCCOLLUM, E. V. The behavior of chickens fed rations restricted to the cereal grains, 57
HOGAN, A. G. Corn as a source of protein and ash for growing animals, 485
Hydrocyanic acid in *Sorghum vulgare*, 25
Hydroxypyridines, structure of curative modifications of, 495
Hyperglycemia and glycosuria, epinephrine, influence of sodium carbonate, 251
—— —— —— after glucose administration, influence of sodium carbonate, 265

INJECTION, timed intravenous, method of, 355
Iodine requirement of pregnant sow, 215

JONES, W., and READ, B. E. Adenine-uracil dinucleotide and the structure of yeast nucleic acid, 111
—— and ——. The mode of nucleotide linkage in yeast nucleic acid, 123

KASTLE, J. H. See BUCKNER and KASTLE, 209
Kernel of oat, dietary deficiencies of, 341
Ketohexose, thiobarbituric acid as qualitative reagent for, 207
KILLIAN, J. A. See MYERS and KILLIAN, 179
KINGSBURY, F. B. The effect of bile and bile salts on the reaction between oleic acid and sodium bicarbonate, 367
KNUDSON, A. See BLOOR and KNUDSON, 7
KOBER, P. A. An improved nephelometer-colorimeter, 155
KURIYAMA, S. The influence of intravenous injection of Witte's peptone upon the sugar content of the blood and epinephrine hyperglycemia and glycosuria, 127

LIVER, glycogen content, relation to diet, 255
LUDEN, G. Studies on cholesterol. III. The influence of bile derivatives in Bloor's cholesterol determination, 463
LYMAN, H. A rapid method for determining calcium in blood and milk, 169
LYMAN, J. F., and TRIMBY, J. C. The excretion of creatine and creatinine parenterally introduced, 1

MARSHALL, M. J. See RUTTAN and MARSHALL, 319
MCCOLLUM, E. V., SIMMONDS, N., and PITZ, W. The dietary deficiencies of the white bean (*Phaseolus vulgaris*), 521
——, ——, and ——. The nature of the dietary deficiencies of the oat kernel, 341
——. See HART, HALPIN, and MCCOLLUM, 57

McDANELL, L., and UNDERHILL, F. P. Studies in carbohydrate metabolism. XIV. The influence of alkali administration upon blood sugar content in relation to the acid-base-producing properties of the diet, 227. XV. The influence of acid-forming and base-forming diets upon blood sugar content, 233. XVI. The relation of epinephrine glycosuria to dosage and to the character of the diet, 245. XVII. Further experiments upon the influence of the intravenous injection of sodium carbonate upon epinephrine hyperglycemia and glycosuria, 251. XVIII. The relation of diet to the glycogen content of the liver, 255. XIX. The influence of the intravenous injection of sodium carbonate upon the hyperglycemia and glycosuria following the subcutaneous administration of glucose, 265. XX. New experiments upon the mechanism of salt glycosuria, 273

MENDEL, L. B. See OSBORNE and MENDEL, 69, 289

Metabolism, carbohydrate, 227, 233, 245, 251, 255, 265, 273

Milk and blood, rapid method for determining calcium in, 169

——, human, composition of, during first 11 days after parturition, 381

MILLER, C. W. See TAYLOR, MILLER, and SWEET, 425

MYERS, V. C., and KILLIAN, J. A. Studies in animal diastases. I. The increased diastatic activity of the blood in diabetes and nephritis, 179

NEPHELOMETER - colorimeter, 155

Nephritis and diabetes, increased diastatic activity of blood in, 179

NEUWIRTH, I. The hourly elimination of certain urinary constituents during brief fasts, 477

Nitrogen, amino-acid, content of blood of various species, 191

——, non-protein, partition of, in blood of fresh water fish, 405

Nucleic acid of yeast, nucleotide linkage in, 123

——————————, structure of, 111

Nucleotide linkage in yeast nucleic acid, 123

OAT kernel, dietary deficiencies of, 341

Oleic acid and sodium bicarbonate, bile and bile salts in reaction of, 367

OSBORNE, T. B., and MENDEL, L. B. The relative value of certain proteins and protein concentrates as supplements to corn gluten, 69

—— and ——. The use of cotton seed as food, 289

PALMER, H. E. See PHELPS and PALMER, 199

Parturition, composition of human milk during first 11 days after, 381

Peptone, Witte's, influence of intravenous injection of, upon sugar content of blood and epinephrine hyperglycemia and glycosuria, 127

Permeability, direct determinations of, 453

Phaseolus vulgaris (white bean), dietary deficiencies of, 321

PHELPS, I. K., and PALMER, H. E. The separation and estimation of butyric acid in biological products. I, 199
Phosphatides in corpus luteum of sow during pregnancy, variation in amount of, 141
PITZ, W. See MCCOLLUM, SIMMONDS, and PITZ, 341, 521
Placenta, chemical composition, 19
PLAISANCE, G. P. Thiobarbituric acid as a qualitative reagent for ketohexose, 207
Plant embryos, isolated, growth of, 209
Plasma and whole blood, creatine and creatinine in, 413
PLASS, E. D. See WILSON and PLASS, 413
Pregnancy, variation of phosphatides in corpus luteum of sow during, 141
Pregnant sow, iodine requirement, 215
Protein and ash for growing animals, corn as source of, 485
——, non-, nitrogen, partition of, in blood of fresh water fish, 405
Proteins and protein concentrates, relative value as supplements to corn gluten, 69
Proteinuria, Bence-Jones, 425

READ, B. E. See JONES and READ, 111, 123
RUTTAN, R. F., and MARSHALL, M. J. The composition of adipocere, 319

SALT glycosuria mechanism, 273
Salts of bile in reaction of oleic acid and sodium bicarbonate, 367
SEIDELL, A. The vitamine content of brewers' yeast, 145
SIMMONDS, N. See MCCOLLUM, SIMMONDS, and PITZ, 341, 521
SMITH, G. E. Fetal athyrosis. A study of the iodine requirement of the pregnant sow, 215
Sodium bicarbonate and oleic acid, bile and bile salts in reaction of, 367
—— carbonate, influence on epinephrine hyperglycemia and glycosuria, 251
—— ——, —— —— hyperglycemia and glycosuria after glucose administration, 265
Sorghum vulgare, effect of anesthetics and frosting on cyanogenetic compounds of, 37
—— ——, hydrocyanic acid in, 25
Sugar of blood, influence of acid-base-producing diet on, 233
—— —— ——, —— —— alkali administration on, 227
—— content of blood and epinephrine hyperglycemia and glycosuria, influence of intravenous injection of Witte's peptone upon, 127
Sulfur, total, in urine, note on Benedict's method for estimation of, 15
SWEET, J. E. See TAYLOR, MILLER, and SWEET, 425

TABES dorsalis, calcium content of cerebrospinal fluid in, 337
TAYLOR, A. E., MILLER, C. W., and SWEET, J. E. Studies in Bence-Jones proteinuria. II, 425
TAYLOR, J. See BRADLEY and TAYLOR, 281
Thiobarbituric acid as qualitative reagent for ketohexose, 207
TRIMBY, J. C. See LYMAN and TRIMBY, 1

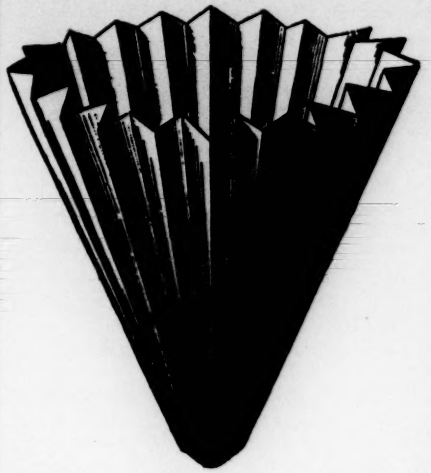
UNDERHILL, F. P. See MCDANELL and UNDERHILL, 227 233, 245, 251, 255, 265, 273

Urea of blood, concentration of, effect on rate of urea excretion, 391
—— excretion, rate of, 391, 399
Urinary constituents, hourly elimination during brief fasts, 477
Urine, colorimetric determination of ammonia in, 329
——, note on Benedict's method for estimation of total sulfur in, 15
—— volume and rate of urea excretion, 399

VITAMINE content of brewers' yeast, 145
Vitamines, chemical nature of, 495

WATANABE, C. K. See ADDIS and WATANABE, 391, 399
WILLAMAN, J. J. The effect of anesthetics and of frosting on the cyanogenetic compounds of *Sorghum vulgare*, 37
——. The estimation of hydrocyanic acid and the probable form in which it occurs in *Sorghum vulgare*, 25
WILLIAMS, R. R. The chemical nature of the vitamines. III. The structure of the curative modifications of the hydroxypyridines, 495
WILSON, D. W., and ADOLPH, E. F. The partition of non-protein nitrogen in the blood of fresh water fish, 405
—— and PLASS, E. D. Creatine and creatinine in whole blood and plasma, 413
WODEHOUSE, R. P. Direct determinations of permeability, 453
WOODYATT, R. T. The method of timed intravenous injections, 355

YEAST, brewers', vitamine content of, 145
—— nucleic acid, nucleotide linkage in, 123
—— —— ——, structure of, 111



FOLDED FILTERS

GRUEBLER STAINS
MERCK'S—J. T. BAKER'S
B. & A. Standard Chemicals,
American Glassware and
Porcelain Ware.

ANY and ALL LABORATORY REQUIREMENTS CARRIED IN ABUNDANCE. WE can give your orders immediate and intelligent attention.

LABORATORY SUPPLIES AND CHEMICALS

DAIGGER

54 West Kinzie Street

CHICAGO ILLINOIS

INTERNATIONAL CENTRIFUGES

INTERNATIONAL EQUIPMENT COMPANY

352 Western Ave. (Brighton)
BOSTON, MASS.

Catalog Cy. on request

GUINEA PIGS

WE supply healthy stock for laboratory use on yearly contracts or small lots. Prices quoted f. o. b. destination. Safe arrival guaranteed.

HOAG & HUNTLY
Springport, Mich.

THE JOURNAL OF IMMUNOLOGY

Official Organ of the Society for Serology and Hematology and the American Association of Immunologists

ARTHUR F. COCA, *Editor*

The Journal is of interest from the practical and theoretical standpoints.
Five dollars a volume—United States and Canada. Five dollars and fifty cents elsewhere.

Order from WILLIAMS & WILKINS CO.
Baltimore, U. S. A.